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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP HPLC METHOD FOR ESTIMATION OF DARUNAVIR AND ITS RELATED SUBSTANCE IN TABLET DOSAGE FORM

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ABSTRACT

To develop simple, precise, rapid and accurate reverse phase HPLC method for estimation of darunavir and its related dosage form in tablet dosage form. The adequate chromatographic separation was performed on Inertsil C8 column (250mm×4.6mm) 5µm using gradient elution. Other HPLC parameter which were optimised flow rate 1.5 ml/min; the effluent was monitored at 265 nm, column oven temperature 50ºC injection volume 5 µl and run time was 80min The development method was statistically validated for linearity (1-4.5 ppm). Forced degradation study revealed that drug was sensitive to Acid, Base, Peroxide degradation, in other condition not adequate degradation observed. The accuracy of the present method was evaluated at 50%, 100%, 150% was found to be within limits 80-101%.Precision studies were carried out and RSD values were less than 5. The Method was found to be robust. The proposed method was found to be specific, accurate, precise and linear can be used for estimation of darunavir in tablet dosage form.

KEYWORDS: RP HPLC, Darunavir, Pharmaceutical dosage form.

INTRODUCTION

Darunavir is chemically (3R, 3aS, 6aR)-hexahydrofuro [2,3-b] furan-3-ylN-[(2S,3R)-3-hydro -xyl-4-[N-(2methylpropyl)4-aminobenzenesulfonamido]-1-phenylbutan-2-yl]carbamate.[1] Darunavir molecular formula is $C_{27}H_{37}N_3O_7$. Molecular weight is 547.664 g /mol.[2] It is a amorphous white, solid, freely soluble in methanol, acetonitrile and soluble in ethanol.[3] Darunavir is a protease inhibitor (anti-retroviral drug).[4] Darunavir inhibits HIV protease enzyme which give cleavage of HIV encoded Gag-Pol poly proteins complex in virus-infected cells, which prevents the formation of mature non - infectious virus particles.[5]

Literature surveys reveal that various analytical methods have been reported for Darunavir by UV Spectroscopy,[6] RP HPLC,[7] HPTLC,[8] LC-MS/MS.[9] However Literature search reveal methods are used to ensure Stability and estimation of darunavir.[10] Method such as Method development and stability study by chromatographic method for perampanel in API and tablet dosage form.[11] So here attempt will be made to develop and validate simple, precise, accurate RP HPLC method for estimation of darunavir and its related substance in Tablet dosage form. [12]

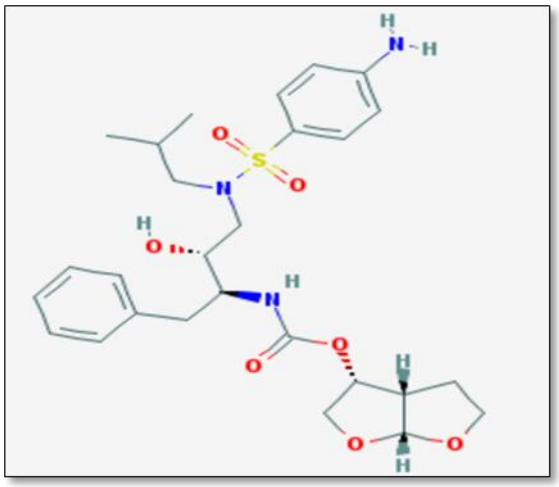

Figure 1: Structure of Darunavir.

MATERIALS AND METHODS

Material and Reagents

Drugs, chemicals and solvents: Acetonitrile, di Potassium Hydrogen Phosphate, di Ammonium hydrogen phosphate, Ortho Phosphosphoric Acid, Hydrochloric acid, Sodium

hydroxide, Hydrogen peroxide were procured from Merck and Finar Reagent. Darunavir standard Gifted by Zydus Cadila Health Care, Ltd., Moraiya, Ahmedabad.

Instrument

Shimadzu UV-1800 UV-Visible spectrophotometer was employed with spectral bandwidth of 1nm attached to computer loaded with shimadzu 1200Series with PC software. Analytical Balance Mettler Toledo, pH meter Lab India Up Controlled and Sonicater Digital Ultra Sonicater were used for the study.

FINAL OPTIMIZATION METHOD

Mobile phase A: Buffer (100 %)

Mobile phase B: Buffer: ACN (15: 85 %)

Chromatographic condition:

Column: Inertsil C8 (250mm×4.6mm) 5µm

Flow rate: 1.5 ml/min

Injection volume: 5µl

Column temperature: 50°C

Table 1: Gradient Program.

Sr. No.	Time	Mobile Phase A	Mobile Phase B
1	0	70	30
2	15	60	40
3	55	52	48
4	60	52	48
5	70	05	95
6	73	70	30
7	80	70	30

Preparation of Buffer

Weigh accurately about 6.8gm potassium dihydrogen phosphate in 5000 ml of water sonicate to dissolve and mixed. Adjust the pH 4.0 ± 0.05 with diluted Ortho-phosphoric acid and filter through 0.45 µ PVDF membrane filter paper.

Preparation of mobile phase

Mobile phase A made by Buffer 100 % v/v and Mobile phase B made by take Buffer: Acetonitrile (15:85% v/v) and sonicate it to dissolve. Filter through 0.45 µ PVDF membrane filter paper.

Diluent

Take water 30 ml, Acetonitrile 70 ml and Tri ethyl amine 1 ml in 100 ml flask. Adjust 6.2 pH with ortho phosphoric acid. Sonicate it to dissolve. Filter through 0.45 µ PVDF membrane filter paper.

Preparation of Standard Stock Solution: (API)

Weight accurately about 50 mg of Darunavir standard (USPRS) in to a 50 ml volumetric flask add 25 ml of diluent, sonicate to dissolve and make up volume with 0.1 N HCl and mix. Take 5.0 ml and transferred into 50 ml volumetric flask. Make up volume with diluent. Take 5.0 ml from Std. Stock Solution (50 ppm) was transferred into 20 ml of volumetric flask and then diluted with the diluent. (10ppm)

Spiked impurities mixture: (Specification limit of impurities 0.15 %)

20 mg of DNA - Diamino impurity and DNA amino nitro impurity into 100 ml volumetric flask individually. Then add 5 ml of acetonitrile in each flask & sonicate to dissolve impurities. Then make up volume with diluents (200 ppm). Now transfer 7.5 ml of individual impurity in single 50 ml volumetric flask and make up the volume up to mark with diluent and mix well. (30 ppm)

1) DNA stage - 1 Impurity: Weight 20 mg of DNA stage 1 impurity into 100 ml volumetric flask. Add 5 ml of acetonitrile and Sonicated to dissolve it. Make up volume with diluent.
2) DNA-Diamino impurity: Weight 20 mg of DNA-Diamino impurity 100 ml volumetric flask. Add 5 ml of acetonitrile and Sonicated to dissolve it. Make up volume with diluent.
3) DNA Difuranyl Impurity: Weight 20 mg of DNA Difuranyl Impurity into 100 ml volumetric flask. Add 5 ml of acetonitrile and Sonicated to dissolve it. Make up volume with diluent.
4) DNA Amino Nitro Impurity: Weight 20 mg of DNA Amino Nitro Impurity into 100 ml volumetric flask. Add 5 ml of acetonitrile and Sonicated to dissolve it. Make up volume with diluent.
5) DNA Dimer Impurity: Weight 20 mg of DNA Dimer Impurity into 100 ml volumetric flask. Add 5 ml of acetonitrile and Sonicated to dissolve it. Make up volume with diluent.

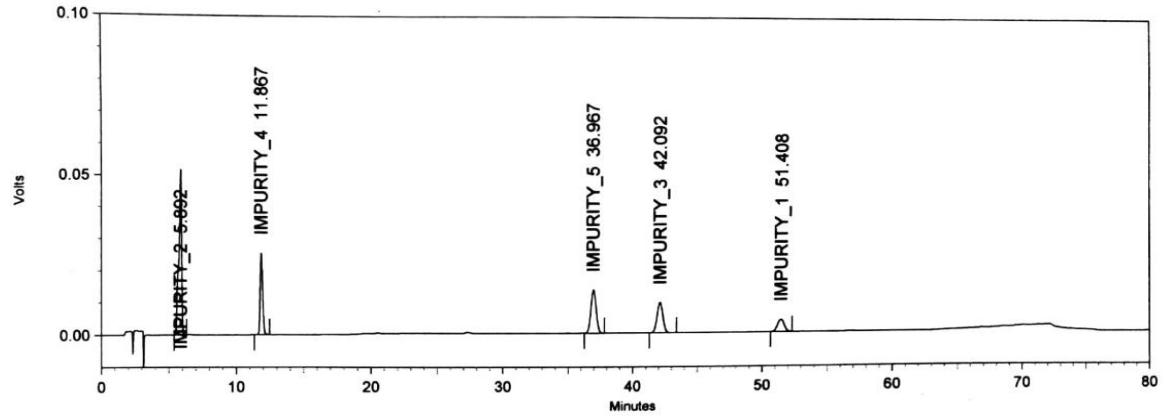

Figure 2: Chromatograph of Impurity Mixture.

Table 2: System suitability parameters in optimized condition.

Peak Name	Retention time (min)	Area	Assymetry	Resolution	Theoritical Plates
Darunavir	27.523	278665	1.28	-	89730
IMP.2	5.836	100.90	0.79	6.71	10455
IMP.3	42.075	72.39	1.05	6.93	6070
IMP.4	11.812	123.58	0.82	6.13	12344
IMP.5	36.933	78.76	0.90	6.67	4982

FORCED DEGRADATION STUDY

Acid degradation

Weight Darunavir crushed powder equivalent to 200 mg [about 293.00 mg] into 100 ml volumetric flask. Add 50 ml of diluent; Sonicated 45 min. Add 2 ml 5 N HCl, Placed into 80°C containing water bath for 30 min. Cool Neutralize with 2 ml of 5 N NaOH. Mix well to dissolve. Make up volume with diluent. Filter with 0.45µ PVDF filter.

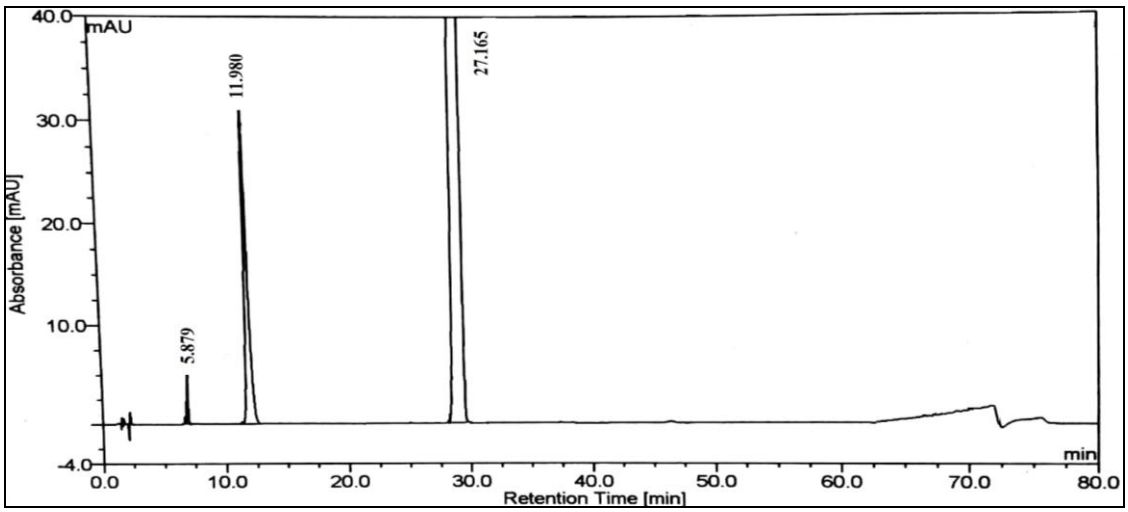

Figure 3: Chromatogram of Acid Degradation.

Base degradation

Weight Darunavir crushed powder equivalent to 200 mg. [about 293.00 mg] into 100 ml volumetric flask. Add 50 ml of diluent; Sonicated 45 min. Add 2 ml 5 N NaOH, Placed into 80°C containing water bath for 30 min. Cool Neutralize with 2 ml of 5 N HCl. Mix well to dissolve. Make up volume with diluent. Filter with 0.45µ PVDF filter.

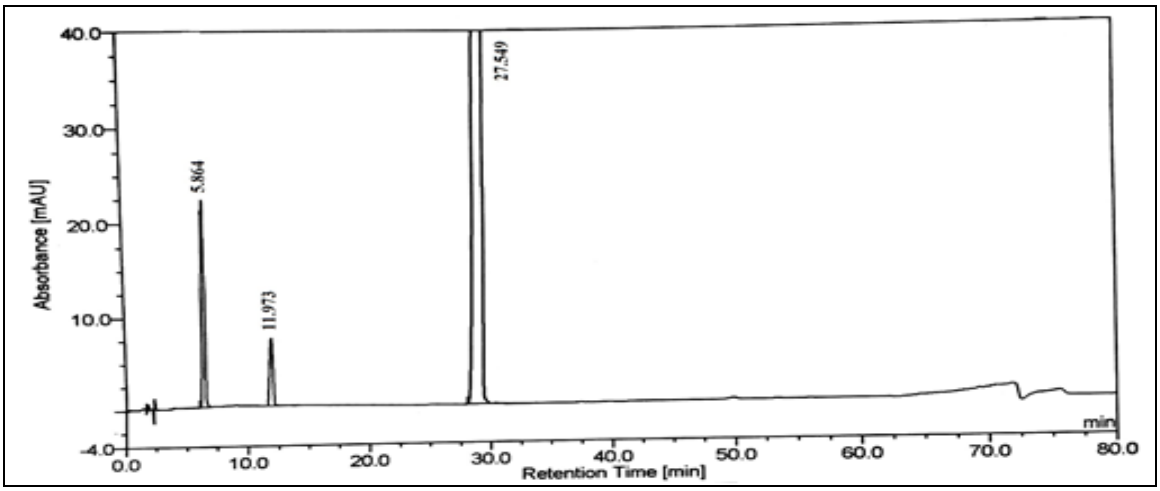

Figure 4: Chromatogram of Base Degradation.

Peroxide degradation

Weight Darunavir granules powder equivalent to 200 mg. [about 293.00 mg] into 100 ml volumetric flask. Add 50 ml of diluent. sonicated 60 min. Add 2 ml of 10 % H2O2 into this and placed into 60°Cwater bath for 60 minutes. Cool & make up volume with diluent. Filter with diluent. Filter with 0.45µ PVDF filter.

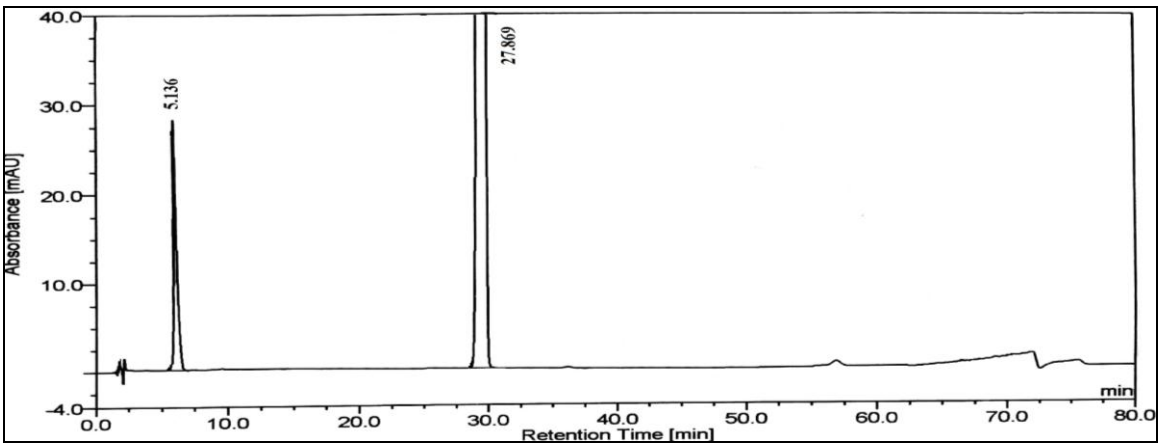

Figure 5: Chromatogram of Peroxide Degradation.

Table 3: Calculation for degradation.

Sr. No.	Stress Condition	Duration	Area	% Degradation	% Mass Balance
1	Acid Hydrolysis (5N HCl_2mL)	80°C for 30 min.	23566.67	15.01	94.81
2	Base Hydrolysis (5N NaoH_2mL)	80°C for 30 min.	24602.84	11.2	95.24
3	Peroxide degradation (10%H2O2_2mL)	60°C for 60 min.	25417.88	8.33	98.76

METHOD VALIDATION

Linearity

According to specification limit, impurity should not be present more than 0.15%, so solutions of linearity of impurity prepared as following in which0.15 % of sample concentration considered as 100 % that is 30 ppm (2000 ppm is sample concentration). And from that consideration LOD, 50%, 80%, 100%, 120%, and 150 % prepare.

Table 4: Linearity result of DNA-Diamino Impurity.

Level	Concentration (µg/ml)	Area
LOQ	1	30584
50 %	1.5	44974
80 %	2.4	69496
100 %	3	87695
120 %	3.6	102850
150 %	4.5	126539

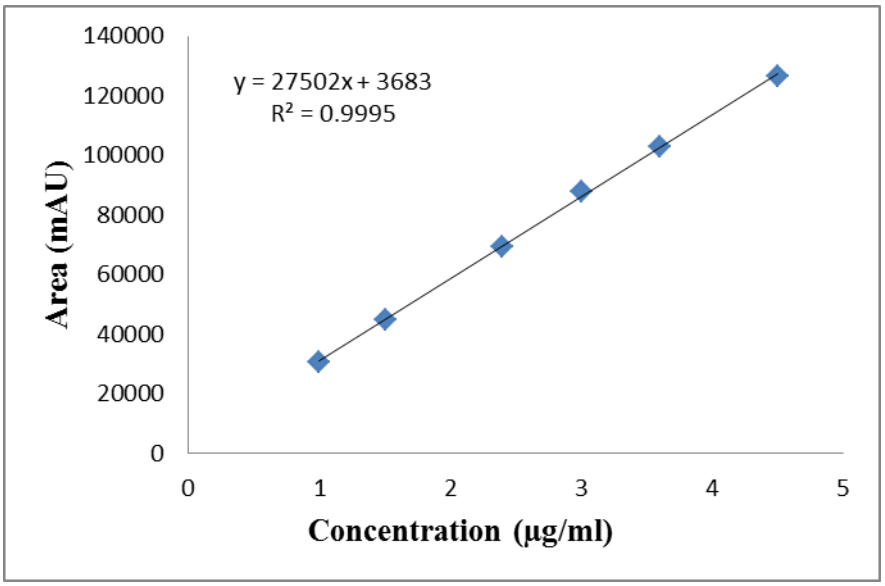

Figure 6: Linearity curve DNA-Diamino Impurity.

Table 5: Linearity result of DNA Amino Nitro Impurity.

Level	Concentration (µg/ml)	Area
LOQ	1	30541
50 %	1.5	44958
80 %	2.4	70925
100 %	3	87452
120 %	3.6	102761
150 %	4.5	125945

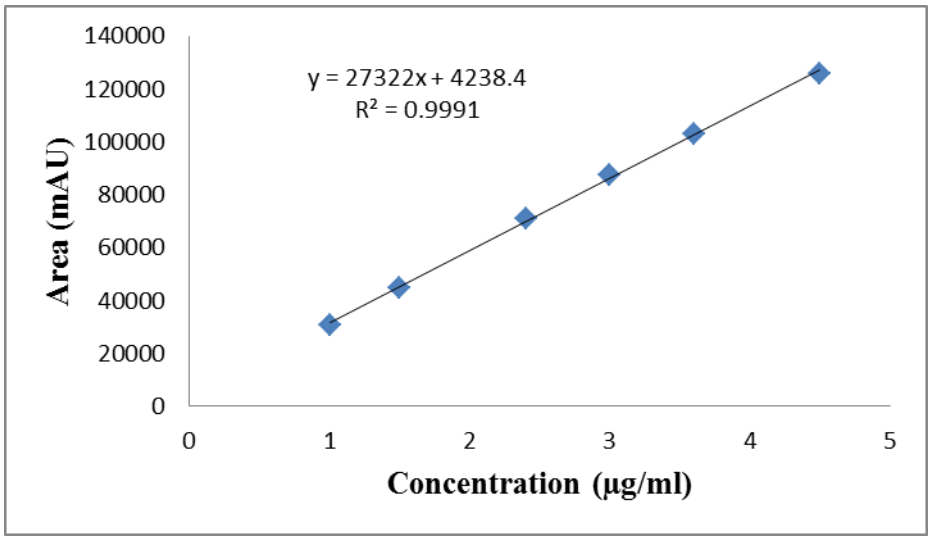

Figure 7: Linearity curve DNA Amino Nitro Impurity.

Accuracy

According to specification limit of all impurities which is not more than 0.15%, amount of individual impurity will be 30 ppm which is 0.15 % of 2000 ppm (sample concentration). Accuracy is performed in sample at 4 levels which are LOQ, 50%, 100% and 150% in three sets.

Table 6: Recovery result of DNA-Diamino Impurity.

Set	Level	Amount added	Area	Amount Found	%Recovery	%Mean Recovery	*SD	*RSD
1	50%	1.5	35512	1.252	83.46			
2	50%	1.5	35678	1.258	83.73	83.95	0.63	0.75
3	50%	1.5	36025	1.270	84.66			
1	100%	3	84472	2.979	99.3			
2	100%	3	85298	3.00	100.26	99.46	0.73	1.35
3	100%	3	84072	2.965	98.83			
1	150%	4.5	128428	4.530	100.66			
2	150%	4.5	128396	4.529	100.64	100.32	0.57	0.94
3	150%	4.5	127165	4.485	99.66			

***** *SD-Standard Deviation, *RSD- Relative Standard Deviation.*

Table 7: Recovery result of DNA Amino Nitro Impurity.

Set	Level	Amount added	Area	Amount Found	%Recovery	%Mean Recovery	*SD	*RSD
1	50%	1.5	35527	1.253	83.53			
2	50%	1.5	36535	1.288	85.86	84.71	1.16	1.37
3	50%	1.5	36056	1.271	84.73			
1	100%	3	83596	2.948	98.26			
2	100%	3	84563	2.982	99.4	99.42	1.16	1.17
3	100%	3	85572	3.018	100.6			
1	150%	4.5	128684	4.539	100.8			
2	150%	4.5	127564	4.499	99.97	100.5	0.49	0.49
3	150%	4.5	128595	4.536	100.8			

** SD-Standard Deviation, RSD- Relative Standard Deviation.*

Precision

According to specification limit of all impurities which is not more than 0.15%, amount of individual impurity will be 30 ppm which is 0.15 % of 2000 ppm (sample concentration). For Repeatability sample containing all impurities at 100% level for six times and for Intermediate precision Sample containing all impurities 50%, 100% and 150% level injected for Interday precision and Intraday precision it is injected in 3 sets.

REPETABILITY

Table 8: Repeatability result of DNA-Diamino impurity.

Sr. No	Area	Mean	*SD	*RSD
1	44591	44594.5	885.5396	1.98
2	45642			
3	44937			
4	43048			
5	45063			
6	44286			

** SD-Standard Deviation, RSD- Relative Standard Deviation.*

Table 9: Repeatability result of DNA Amino Nitro Impurity.

Sr. No	Area	Mean	*SD	*RSD
1	87591	87761.17	1294.7	1.47
2	85642			
3	88937			
4	87048			
5	89063			
6	88286			

** SD-Standard Deviation, RSD- Relative Standard Deviation.*

INTERMEDIATE PRECISION

Intraday Precision

Table 10: Intraday precision result of DNA-Diamino impurity.

For 50 % Level						
Set	**Level**	**Morning**	**Evening**	**Mean**	***SD**	***RSD**
1	50%	44632	44531	44581.5	71.41	0.16
2	50%	45256	44985	45120.5	191.62	0.42
3	50%	44261	43326	43793.5	661.14	1.50
For 100 % Level						
Set	**Level**	**Morning**	**Evening**	**Mean**	**SD**	**RSD**
1	100%	88619	87361	87990	889.54	1.01
2	100%	89153	87593	88373	1103.08	1.24
3	100%	87123	88376	87749.5	886.00	1.00
For 150 % Level						
Set	**Level**	**Morning**	**Evening**	**Mean**	**SD**	**RSD**
1	150%	121462	119168	120315	1622.10	1.34
2	150%	129554	132518	131036	2095.86	1.59
3	150%	125389	123128	124258.5	1598.76	1.28

***** *SD-Standard Deviation, RSD- Relative Standard Deviation.*

Table 11: Intraday precision result of DNA Amino Nitro Impurity.

For 50 % Level						
Set	**Level**	**Morning**	**Evening**	**Mean**	***SD**	***RSD**
1	50%	44244	45079	44661.5	590.43	1.32
2	50%	45094	44132	44613	680.23	1.52
3	50%	45142	44375	44758.5	542.35	1.21
For 100 % Level						
Set	**Level**	**Morning**	**Evening**	**Mean**	**SD**	**RSD**
1	100%	88957	87238	88097.5	1215.51	1.37
2	100%	87056	88432	87744	972.97	1.10
3	100%	88979	87010	87994.5	1392.29	1.58
For 150 % Level						
Set	**Level**	**Morning**	**Evening**	**Mean**	**SD**	**RSD**
1	150%	128354	132429	130391.5	2881.46	2.20
2	150%	121462	119168	120315	1622.10	1.34
3	150%	125389	121319	123354	2877.92	2.33

***** *SD-Standard Deviation, RSD- Relative Standard Deviation.*

Interday Precision

Table 12: Interday precision result of DNA-Diamino impurity.

For 50 % Level						
Set	**Level**	**Morning**	**Evening**	**Mean**	***SD**	***RSD**
1	50%	44596	44385	44490.5	149.19	0.33
2	50%	44965	45663	45314	493.56	1.08
3	50%	45215	44372	44793.5	596.09	1.33
For 100 % Level						
Set	**Level**	**Morning**	**Evening**	**Mean**	**SD**	**RSD**
1	100%	89265	88462	88863.5	567.80	0.63
2	100%	88042	86715	87378.5	938.33	1.04
3	100%	86485	88743	87614	1596.64	1.82
For 150 % Level						
Set	**Level**	**Morning**	**Evening**	**Mean**	**SD**	**RSD**
1	150%	122362	138668	130515	11530.08	0.05
2	150%	373954	375218	374586	893.78	0.23
3	150%	372789	374028	373408.5	876.10	0.23

***** *SD-Standard Deviation, RSD- Relative Standard Deviation.*

Table 13: Interday precision result of DNA Amino Nitro Impurity.

For 50 % Level						
Set	**Level**	**Morning**	**Evening**	**Mean**	***SD**	***RSD**
1	50%	44785	45743	45264	677.40	1.49
2	50%	45024	45543	45283.5	366.98	0.81
3	50%	44248	45108	44678	608.11	1.36
For 100 % Level						
Set	**Level**	**Morning**	**Evening**	**Mean**	**SD**	**RSD**
1	100%	81243	79848	80545.5	986.41	1.22
2	100%	80156	81946	81051	1265.72	1.56
3	100%	80953	78456	79704.5	1765.64	2.21
For 150 % Level						
Set	**Level**	**Morning**	**Evening**	**Mean**	**SD**	**RSD**
1	150%	123462	118995	121228.5	3158.64	2.60
2	150%	115849	112456	114152.5	2399.21	2.10
3	150%	116076	114048	115062	1434.01	1.24

***** *SD-Standard Deviation, RSD- Relative Standard Deviation.*

Robustness

According to robustness, there is minor deliberate change made in chromatographic parameter with reference of Flow rate and Column temperature. To observe robustness, 100% level solution used.

Change in Flow Rate

Inject the solution of 100% level of all impurities at flow rate of 1.3 ml/min, and 1.7 ml/min and calculate RSD for all the responses of all impurities individually.

Change in Column Tempreture

Inject the solution of 100% level of all impurities at Column temperature of 48°C, and 52°C and calculate RSD for all the responses of all impurities individually.

Table 14: Robustness result of 3-Amino salicylic acid DNA-Diamino impurity.

Parameter	Change	Area	Mean	*SD	*RSD
Flow rate (ml/min)	1.3	86238	86708.3	863.867	0.99
	1.3	87569			
	1.3	88216			
	1.5	87498			
	1.5	86420			
	1.5	85685			
	1.7	86204			
	1.7	85856			
	1.7	86689			
Column Tempreture	48	88686	80153	1053.55	1.31
	48	87998			
	48	86951			
	50	81056			
	50	80047			
	50	81012			
	52	84842			
	52	86476			
	52	85842			

***** *SD-Standard Deviation, RSD- Relative Standard Deviation.*

Table 15: Robustness result of DNA Amino Nitro Impurity.

Parameter	Change	Area	Mean	*SD	*RSD
Flow rate (ml/min)	1.3	78325	80272.2	1197.718	1.49
	1.3	81423			
	1.3	80388			
	1.5	80989			
	1.5	81852			
	1.5	79952			
	1.7	78512			
	1.7	80624			
	1.7	80385			
Column Temperature	48	79843	80153	1053.558	1.31
	48	81462			
	48	80354			

	50	81056			
	50	80047			
	50	81012			
	52	79234			
	52	80356			
	52	78013			

***** *SD-Standard Deviation, RSD- Relative Standard Deviation.*

CONCLUSION

All the parameter and results were found within the acceptance limit as given in the Validation protocol. So we can conclude that developed RP-HPLC method was Selective, specific, sensitive, linear, accurate, precise, and robustness. Therefore method is found to be specific for Darunavir related substances with good resolution. It can be applied for the forced degradation study. So the proposed method can be used in pharmaceutical analysis for Forced degradation study and routine quality control sample of Darunavir Tablet.

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