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GRADIENT HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR DETERMINATION OF RELATED SUBSTANCES IN [(3aS, 4R, 6aR)-2, 3, 3a, 4, 5, 6a-HEXAHYDROFURO [2, 3-b] FURAN-4-YL] N-[(2S, 3R)-4-[(4-AMINOPHENYL) SULFONYL-(2-METHYLPROPYL) AMINO]-3-HYDROXY-1-PHENYLBUTAN-2YL] CARBAMATE DOSAGE FORM

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Keywords:

RP-HPLC, Forced degradation, Darunavir

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ABSTRACT: A simple, selective, linear, precise and accurate RP-HPLC method was developed and validated for quantification of Darunavir in bulk and its tablet dosage form. The separation was carried out on Zorbax Eclipse XDB C18 (150 × 4.6 mm; 5 µm) column at 35 °C temperature using ammonium acetate buffer as mobile phase-A, acetonitrile (100%) as mobile phase-B. The flow rate was 1.0 ml/min and effluent was detected at 265 nm. The retention time of Darunavir propylene glycolate was 31.34 min. The percentage recovery was within the range between 95.46% and 100.17% for Darunavir propylene glycolate. The linear ranges were found to be 0.086-3.084 µg/ml (r^2 = 0.9997) for Darunavir propylene glycolate. The percentage relative standard deviation for accuracy and precision was found to be less than 5.0%. Hence, the method could be successfully applied for routine analysis of Darunavir in pharmaceutical formulations.

INTRODUCTION: Darunavir is a white to off-white hygroscopic powder. Its solubility in organic solvents varies significantly and it is very slightly soluble in aqueous solution (solubility increases with decreasing pH). Therefore, the particle size is likely to be important to the rate and possibly to the extent of absorption of Darunavir. It contains 5 chiral centers, however the manufacturing process leads, in a consistent way, to the single enantiomer 3R, 3aS, 6aR, 1S, 2R [1-2].

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The absolute configuration has been confirmed by X-ray diffraction analysis. Under commercial synthesis conditions, darunavir is isolated as a crystalline ethanolate (1:1 solvate). It can exist as a non-solvated amorphous form and as a hydrate form as well Darunavir 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. It is used under the brand name of Prezista and it is used with other combination drug like Darunavir ethanolate, cobicistat, ritonavir but a few combinations give good results in the treatment of HIV.

MATERIALS AND METHODS:

Instrumental and Analytical Conditions:

Reagents and Chemicals: The drug Darunavir substances and its process related impurities were

gifted by Active Pharma Labs. (Hyderabad, India). Buffer salts were purchased from Merck and Sigma Aldrich, India. Highly purified water for HPLC was obtained from Milli Q plus water purifying system, Millipore. Methanol and acetonitrile of HPLC grade were obtained from RANKEM, India. Mobile phase was vacuum filtered through a 0.45µm Polyvinyl Dene Fluoride (PVDF) filter membrane and degassed using a sonicator to remove the dissolved gases.

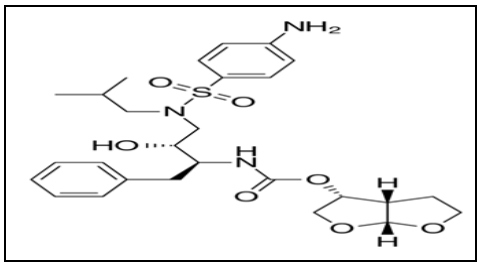

FIG. 1: CHEMICAL STRUCTURE OF DARUNAVIR

Equipment: Waters e2695 gradient system with Empower-3 software and 2996 module Photo Diode Array detectors equipped with a quaternary solvent delivery pump, automatic sample injector and column thermostat was used for the analysis.

Chromatographic Conditions:

Column : Zorbax Eclipse XDB C18 (150 mm × 4.6mm), 5µ or equivalent
Pump mode : Gradient
Flow rate : 1.0 mL/min.
Detection : UV, 265 nm.
Injection volume : 20 µL.
Column oven temp : 35 °C
Sample cooler temp : 6 °C
Date acquisition time : 55 min

TABLE 1: GRADIENT PROGRAMME

Time (min)	Solution A	Solution B
0.01	75	25
30	65	35
45	30	70
55	30	70
56	75	25
70	75	25

Preparation of Solutions:

System Suitability Solution: Dissolve 1.6 mg of Darunavir enriched with cyclic carbamate reference sample in 1 ml of diluent.

Standard Solution: Weigh and transfer about 57 mg of Darunavir propylene glycol ate working standard (equivalent to about 50 mg of darunavir) in to a 50 mL clean, dry volumetric flask, add about 35 mL of diluent and sonicate to dissolve. Dilute to volume with diluent and mix. Diluent 5 mL of this solution to 50 mL with diluent and mix. Dilute 5 mL of this solution to 100 mL with diluent and mix. Filter the solution through Millipore PVDF 0.45 µm membrane filter or suitable [5-6].

Sample Solution: Weigh and finely powder not less than 10 tablets using a suitable mortar and pestle. Weigh and transfer tablet powder equivalent to about 200 mg of Darunavir in to a 200 mL clean, dry volumetric flask. Add about 150 mL of diluent and sonicate for 30 min at room temperature with intermittent shaking at about each 5 min interval. Dilute to volume with diluent and mix. Filter the solution through Millipore PVDF 0.45 µm membrane filter or suitable [7].

Placebo Solution: Weigh and transfer placebo powder equivalent to about 50 mg of Darunavir into a 50 mL clean, dry volumetric flask. Add about 30 mL of diluent and sonicate for 30 min at room temperature with intermittent shaking at about each 5 min interval. Dilute to volume with diluent and mix. Filter the solution through Millipore PVDF 0.45 µm /mdi Nylon 0.45 µm membrane filter or suitable [8].

TABLE 2: ELUTION ORDER

S. no.	Name	RRT	Impurity Classification
1	Diamino Alcohol	0.35	Process/Degradant
2	n-Propyl analog	0.75	Process
3	Cyclic Carbamate	0.93	Process/Degradant
4	Darunavir	1.00	-
5	(1S,2S)(3R,3AS,6aR)-stereo isomer	1.10	Process
6	N-Bis THF Darunavir	1.18	Process
7	O-Bis THF Darunavir	1.21	Process

Method Validation: The above method was validated according to ICH guidelines to establish the performance characteristics of a method (expressed in terms of analytical parameters) to meet the requirements for the intended application of the method [9].

Validation Parameters: [10-12]

1. System suitability
2. System & method precision
3. Intermediate precision

4. LOD and LOQ
5. Linearity
6. Robustness
7. Specificity

Spectrum Index for All impurity Mix: Injected all impurity mix solution in to HPLC and pick the spectrums of all impurities **Fig. 2**.

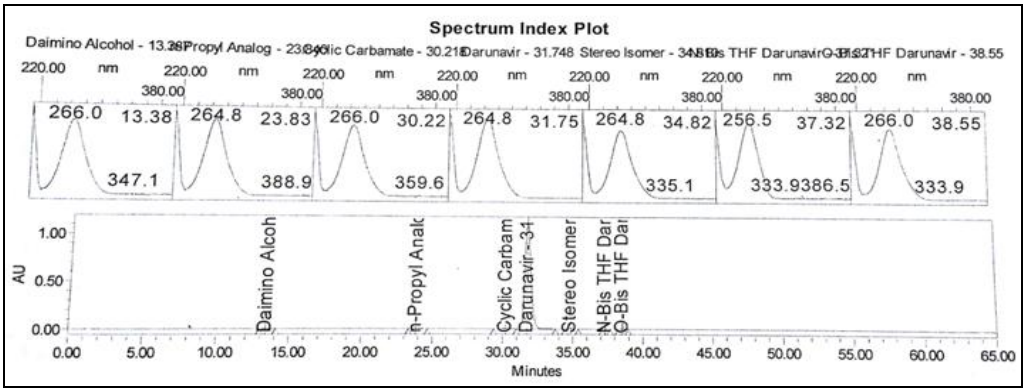

FIG. 2: SPECTRUM INDEX

Conclusion: As per spectrum index the ƛ(Lamda) max concluded 265nm.

System Suitability Solution: Dissolve 1.6 mg of Darunavir enriched with cyclic carbamate reference sample in 1 ml of diluent. All the system suitability parameters are within range and satisfactory as per ICH guidelines [6-8].

System Precision:

Preparation of Solution: Weigh and transfer about 57 mg of Darunavir propylene glycolate working standard into a 50 mL of clean, dry volumetric flask, add about 35 mL of diluent and sonicate to dissolve. Dilute to volume with diluent and mix. Dilute 5 mL of this solution to 50 mL of with diluent and mix. Dilute 5 mL of this solution to 100 mL with diluent and mix (Concentration: about 5 ppm). The standard solution was injected for six times and the areas of chromatograms for all six injections were measured in HPLC. The % RSD for the area of six replicate injections was found to be within the specified limits [9-10].

TABLE 3: SYSTEM SUITABILITY STUDIES OF DARUNAVIR METHOD

S. no.	Name	Retention Time	Area	% Area	USP Resolution
1	Daimino Alcohol	12.05	100487	0.22	
2	n-Propyl Analog	25.22	99132	0.22	28.31
3	Cyclic Carbamate	31.48	105124	0.23	11.35
4	Darunavir	33.38	44914960	98.76	3.55
5	Stereo Isomer	35.83	93609	0.21	6.10
6	N-Bis THF Darunavir	38.02	84604	0.19	8.09

TABLE 4: SYSTEM PRECISION RESULTS FOR DARUNAVIR

S. no.	Injection	Sample Name	Retention Time	Area
1	1	Standard_5ppm	31.34	209934
2	2	Standard_5ppm	31.37	209086
3	3	Standard_5ppm	31.37	209954
4	4	Standard_5ppm	31.34	209628
5	5	Standard_5ppm	31.36	209540
6	6	Standard_5ppm	31.31	210188
		%RSD		0.2

% RSD peak areas of darunavir obtained from six replicate injections of standard solutions should not more than 5.0%.

Conclusion: % RSD of six standard injections was found 0.2.

Method Precision: Six sample solutions were prepared individually using single batch of Darunavir tablets spiked known related substances at specification level and injected into HPLC [9-12].

TABLE 5: METHOD PRECISION RESULTS FOR DARUNAVIR

Name	% m/m					
	Sample-1	Sample-2	Sample-3	Sample-4	Sample-5	Sample-6
Diamino Alcohol	0.196	0.192	0.198	0.195	0.194	0.190
Cyclic Carbamate	0.205	0.200	0.203	0.207	0.203	0.203
Darunavir	0.160	0.160	0.162	0.161	0.160	0.161
Statistical Analysis						
Name	Mean	SD	% RSD		95% Confidence Interval	
Diamino Alcohol	0.194	0.003	1.5		0.003	
Cyclic Carbamate	0.204	0.002	1.0		0.002	
Darunavir	0.161	0.001	0.6		0.001	

TABLE 6: RUGGEDNESS RESULTS FOR DARUNAVIR

Name	% m/m					
	Sample-1	Sample-2	Sample-3	Sample-4	Sample-5	Sample-6
Diamino Alcohol	0.191	0.191	0.190	0.193	0.191	0.191
Cyclic Carbamate	0.191	0.192	0.190	0.193	0.192	0.191
Darunavir	0.165	0.165	0.165	0.164	0.165	0.164
Statistical Analysis						
Name	Mean	SD	% RSD		95%Confidence Interval	
Diamino Alcohol	0.191	0.001	0.5		0.001	
Cyclic Carbamate	0.192	0.001	0.5		0.001	
Darunavir	0.165	0.001	0.6		0.001	

% RSD should not be more than 10.0 for the results of related substances and Darunavir from the six determinations [9-12].

Conclusion: % RSD of all the Impurities was found within the limit. The above results that the test method is precise for the determination of related substances in Darunavir tablets.

% RSD should not be more than 10.0 for the results of Related Substances and Darunavir from the six determinations.

Conclusion: % RSD of all the Impurities was found within the limit.

The above results that the test method is precise for the determination of related substances in Darunavir tablets.

Limit of Detection and Limit of Quantitation: The limit of detection (LOD) and limit of quantitation (LOQ) values of Darunavir and its known related substances were determined using the values of slope, standard deviation and responses of individual analytes that have been obtained from the linearity study carried out from 1% to 150% of specification level for known related substances and for 1% to 150% standard concentration level for Darunavir [11-12].

TABLE 7A: LOD AND LOQ RESULTS FOR DARUNAVIR

Injection ID	Area of Darunavir	
	LOD	LOQ
1	3702	11134
2	3823	11154
3	4002	11251
4	4035	11019
5	3770	11232
6	3922	11316
Statistical Analysis		
Mean	3876	11184
SD	132	105
%RSD	3.4	0.9
Concentration Level		
Concentration(µg/ml)	0.083	0.251
Concentration(µg/ml)	0.008	0.025

TABLE 7B: LOD AND LOQ RESULTS FOR DARUNAVIR

Injection ID	Area of			
	Diamino Alcohol		Cyclic Carbamate	
	LOD	LOQ	LOD	LOQ
1	3803	11151	3647	11801
2	3764	11203	3746	11877
3	3821	11219	3647	11721
4	3828	11240	3687	11811
5	3853	11181	3689	11892
6	3918	11218	3679	11920
	Statistical analysis			
Mean	3831	11202	3683	11837
SD	52	32	36	73
% RSD	1.4	0.3	1.0	0.6
	Concentration Level			
Concentration(µg/ml)	0.066	0.200	0.082	0.250
Concentration(µg/ml)	0.007	0.020	0.008	0.025

Accptance Criteria:

LOD: RSD should not be more than 33.0.
LOQ: RSD should not be more than 10.0%.

Conclusion: The above results within acceptance criteria the above reported LOQ values for Darunavir and its known related substances are below 50% of specification level for known related substances and below 50% of standard concentration level for Darunavir. Hence, the method is precise for the Quantitation of the related substances in Darunavir tablets.

Robustness: Standard and sample solution spiked with known related substance at specification level were prepared as per test method and injected into HPLC at different deliberately varied conditions to evaluate method ability to remain unaffected [5-8].

TABLE 8A: ROBUSTNESS RESULTS FOR DARUNAVIR

Condition	Variation	RT for Darunavir	RT for Diamino alcohol	RT For n-Propyl analog	RT For Cyclic Carbamate	USP Resolution between Cyclic Carbamate and Darunavir
As Such	-	31.40	10.84	23.382	29.31	3.78
Flow	Flow Variation (-0.1)	33.38	12.05	25.23	31.48	3.55
Variation	Flow Variation (+0.1)	30.58	10.35	22.65	28.63	3.50
Column oven	-5°C	32.92	11.163	24.78	31.54	2.54
Temperature	+5°C	30.51	10.97	22.59	28.02	4.48
Mobile Phase	Organic (-2%)	26.90	8.82	19.55	25.13	3.27
	Organic (+2%)	35.42	14.01	28.52	34.35	3.09

TABLE 8B: ROBUSTNESS RESULTS FOR DARUNAVIR

Condition	Variation	RT for Darunavir	RT for Stereo Isomer	USP Resolution between Stereo isomer and Darunavir	RT For N-Bis THF Darunavir	RT For O-Bis THF Darunavir
As Such	-	31.40	34.96	6.53	37.38	38.55
Flow	Flow Variation (-0.1)	33.38	35.83	6.10	38.02	39.16
Variation	Flow Variation (+0.1)	30.58	34.07	7.47	36.78	37.99
Column oven	-5°C	32.92	35.45	6.13	37.57	38.84
Temperature	+5°C	30.51	34.09	7.57	36.99	38.08
Mobile Phase	Organic (-2%)	26.90	30.82	6.98	35.34	36.89
	Organic (+2%)	35.42	37.08	5.67	38.83	39.82

TABLE 8C: ROBUSTNESS RESULTS FOR DARUNAVIR

Condition	Variation	RT for Darunavir	RT for Diamino alcohol	RT For n-Propyl analog	RT For Cyclic Carbamate	Resolution between Cyclic Carbamate and Darunavir
As Such	-	31.40	10.84	23.382	29.31	3.78
Flow	Flow Variation (-0.1)	33.38	12.05	25.23	31.48	3.55
Variation	Flow Variation (+0.1)	30.58	10.35	22.65	28.63	3.50
Column oven	-5°C	32.92	11.163	24.78	31.54	2.54
Temperature	+5°C	30.51	10.97	22.59	28.02	4.48
Mobile Phase	Organic (-2%)	26.90	8.82	19.55	25.13	3.27
	Organic (+2%)	35.42	14.01	28.52	34.35	3.09

The varied conditions include change in flow rate by ± 10%, column oven temperature by ± 5% °C, gradient composition by ± 1% absolute with respect to mobile phase-B, wavelength by ± 5nm, organic composition in mobile phase-A by ± 1 and pH of the buffer ± 0.1 units [8-12].

Conclusion: The system suitability result at each of the varied conditions complied with the requirements as per the test procedure. Also, it was observed from the chromatograms of sample spiked with known related substances obtained from different robust conditions outlined above that, there is no significant variation in relative retention times (RRT) of related substances obtained at each varied conditions.

Hence, it can be concluded that the test method is robust for Related Substances in Darunavir tablets across the extent of changes studied for each of the above parameters.

Recovery:

1. Diamino Alcohol: Potency-96.40%

Stock-1: Weigh and transfer 0.86 mg of Di amino Alcohol impurity in 20 mL clean dry volumetric flask and dilute with Diluent and mix.

Stock-II: Pipette out 5 mL from Stock-I solution in 25 mL and dilute with diluent and mix.

Preparation of Linearity Levels:

5% Level 1- Pipette out 0.50 mL from Stock-II solution in 50 mL and dilute with diluent and mix. Concentration 0.086(µg/mL).

25% Level 2- Pipette out 1.20 mL from Stock-II solution in 25 mL and dilute with diluent and mix. Concentration 0.414(µg/mL)

50% Level 3- Pipette out 1.00 mL from Stock-II solution in 10 mL and dilute with diluent and mix. Concentration 0.863(µg/mL)

100% Level 4- Pipette out 2.00 mL from Stock-II solution in 10 mL and dilute with diluent and mix. Concentration 1.727(µg/mL)

125% Level 5- Pipette out 2.50 mL from Stock-II solution in 10 mL and dilute with diluent and mix. Concentration 2.158(µg/mL)

150% Level 6- Pipette out 3.00 mL from Stock-II solution in 10 mL and dilute with diluent and mix. Concentration 2.590(µg/mL) [9-12]

Concentration (µg/mL) =Weight of impurity/ Dilution × volume taken/ Dilution×Potency/100×1000

TABLE 9A: ACCURACY RESULTS OF DARUNAVIR AND ITS IMPURITIES

Level	Concentration (µg/ml)						
	Diamino Alcohol	Propyl Analog	Darunavir	Cyclic Carbamate	Stereo Isomer	N-Bis THF Darunavir	O-Bis THF Darunavir
5%	0.086	0.100	0.248	0.099	0.0863	0.0909	0.1028
25%	0.414	0.481	1.192	0.477	0.4318	0.4545	0.514
50%	0.863	1.003	2.483	0.993	0.8635	0.909	1.028
100%	1.727	2.005	4.965	1.987	1.7271	1.818	2.056
125%	2.158	2.507	6.206	2.484	2.1588	2.2725	2.5725
150%	2.590	3.008	7.448	2.980	2.5906	2.727	3.084

TABLE 9B: ACCURACY RESULTS OF DARUNAVIR AND ITS IMPURITIES

Level	Area						
	Diamino Alcohol	Propyl Analog	Darunavir	Cyclic Carbamate	Stereo Isomer	N-Bis THF Darunavir	O-Bis THF Darunavir
5%	4366	4574	11058	4738	4228	2920	3405
25%	21230	20411	49627	21874	19405	13869	16274
50%	44595	43927	105842	46714	41709	29503	34605
100%	89851	86971	210398	93294	82336	58748	68978
125%	112936	109359	264078	117699	103328	73774	86567
150%	135344	131088	316283	140215	123482	88084	103400
Slope	52398	43588.4	42504.4	47230.2	47868	32490	33716
CC	0.9999	0.99998	0.9999	0.99997	0.99997	0.99997	0.9999
r^2	0.9994	0.9995	0.9999	0.99994	0.9999	0.9999	0.9999
Y= mx+c	Y=52398-396.2	Y=43588-83.15	Y=42504x-51.43	Y=47230x-247.0	Y=47868x-277.67	Y=32490-308.71	Y=3716x-376.61

Forced Degradation: Forced degradation is a process whereby the natural degradation rate of a product is increased by the application of more stress. Forced degradation studies are used to identify reactions which may occur to degrade a processed product. Long term storage tests are usually used to measure similar properties when final formulations are involved because of the stringent FDA regulations. Degradation of Darunavir was found to occur under acidic condition (2M HCl, 120 min at 85 °C), alkaline condition (2M NaOH, 15 min at RT), oxidative condition (10% H_2O_2, 15 min at 85 °C), photolytic degradation (White Fluorescent light, 1.2 million Lux for 24 h), humidity degradation (90% RH/ 25℃ /120 h) and thermal degradation (the oven at 60 ℃ for 120 h). The developed RP-HPLC method can be used to analyze from its degradation products and hence found to be specific for Darunavir [9].

TABLE 10: FORCED DEGRADATION

Degradation Mechanism	Area of Darunavir	% Degradation	Peak Purity of Darunavir	
			Purity Angle	Purity Thershold
Undegraded Sample	17736767	-	0.029	0.262
Acid Degradation	16374222	7.7	0.028	0.258
Base Degradation	14075985	20.8	0.031	0.253
Peroxide Degradation	16839877	5.1	0.027	0.261
Thermal Degradation	17570122	0.9	0.026	0.261
Photolytic Degradation	17393980	1.9	0.021	0.259
Humidity Degradation	17335620	2.3	0.025	0.258

Acceptance Criteria: Darunavir peak should be homogeneous and there should be no co-eluting peaks. Peak purity of Darunavir peak should pass as per Acceptance criteria [14].

System Suit Chromatogram:

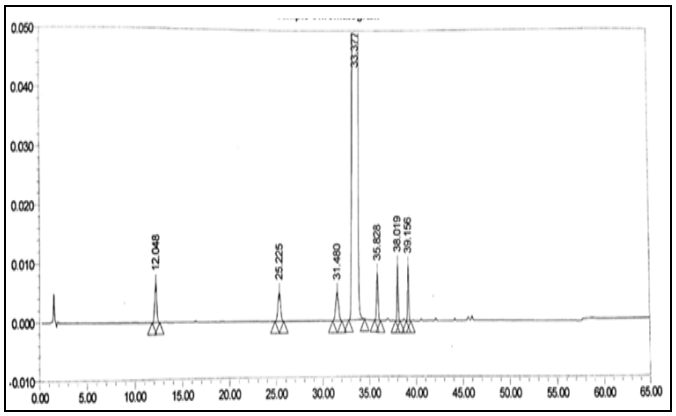

FIG. 3: SYSTEM SUIT CHROMATOGRAM

Linearity:

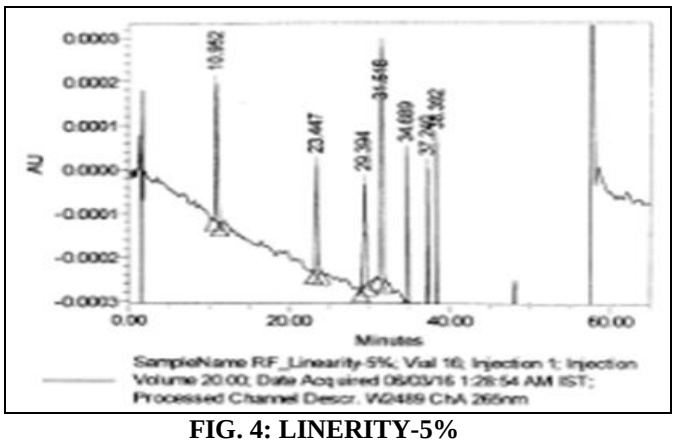

FIG. 4: LINERITY-5%

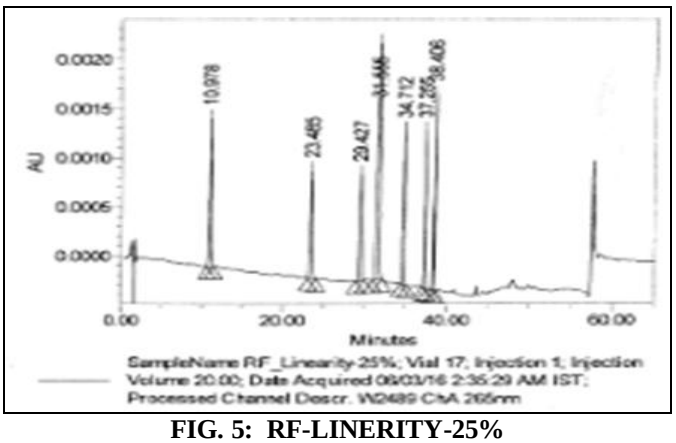

FIG. 5: RF-LINERITY-25%

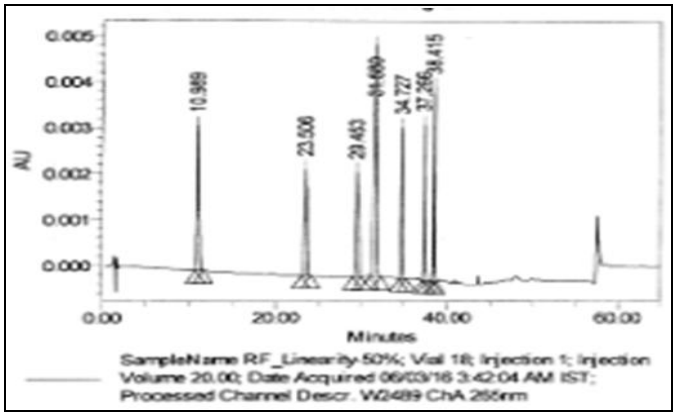

FIG. 6: LINERITY-50%

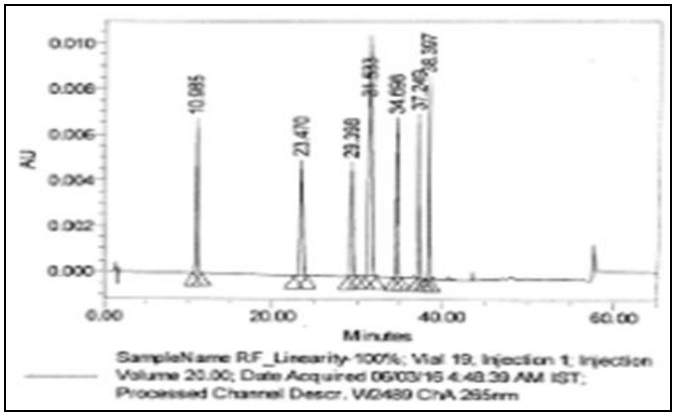

FIG. 7: RF-LINERITY-100%

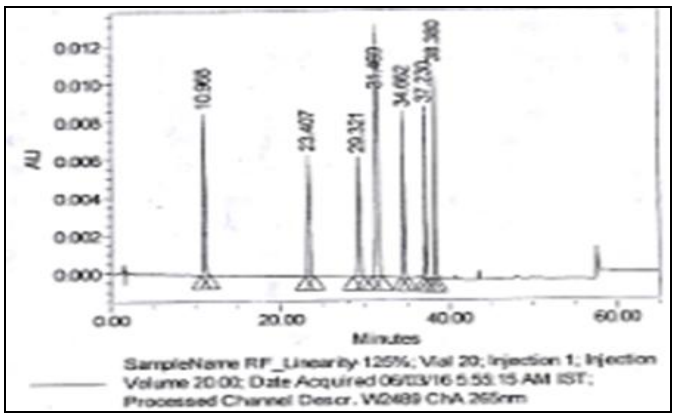

FIG. 8: LINERITY-125%

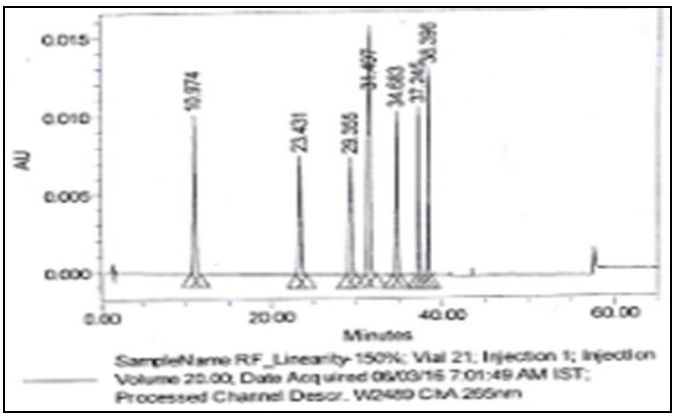

FIG. 9: RF-LINERITY-150%

Calibration Curves:

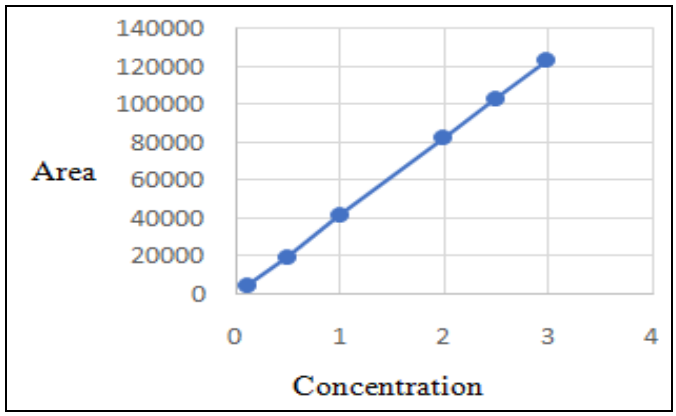

FIG. 10: DIAMINO ALCOHOL

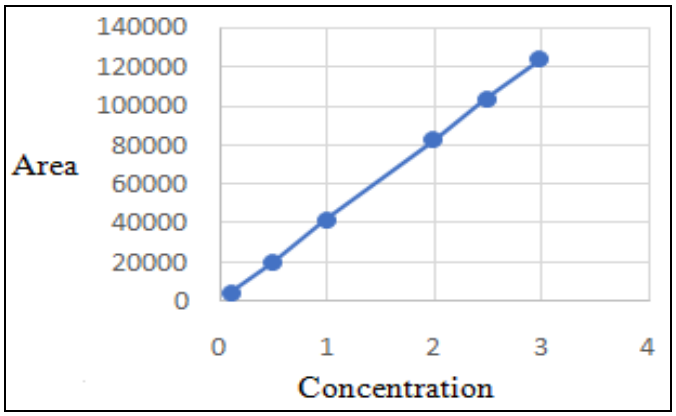

FIG. 11: PROPYL ANALOG

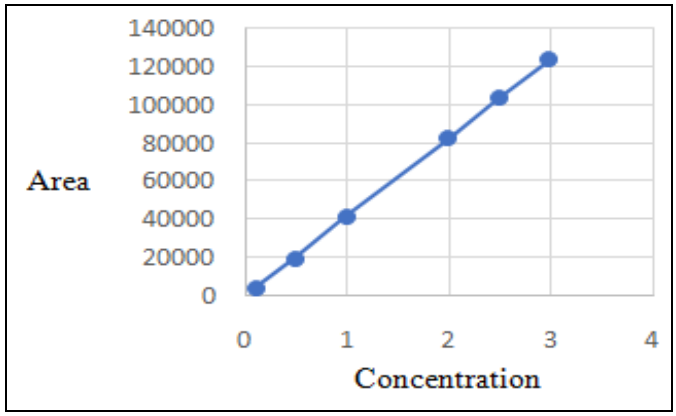

FIG. 12: DARUNAVIR

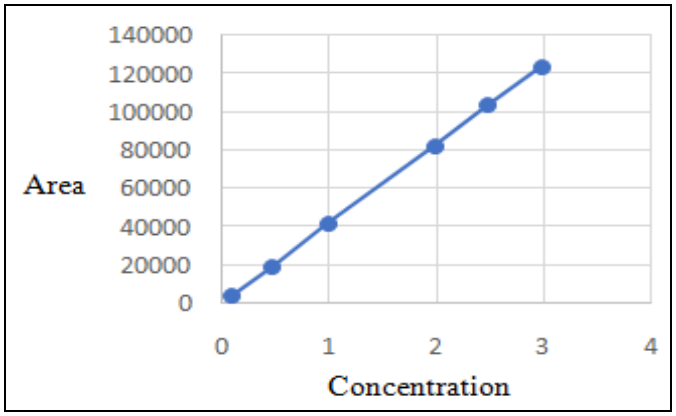

FIG. 13: CYCLIC CARBAMATE

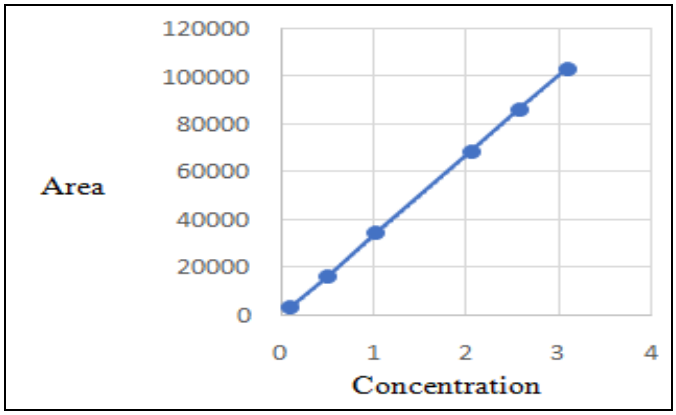

FIG. 14: STEREO ISOMER

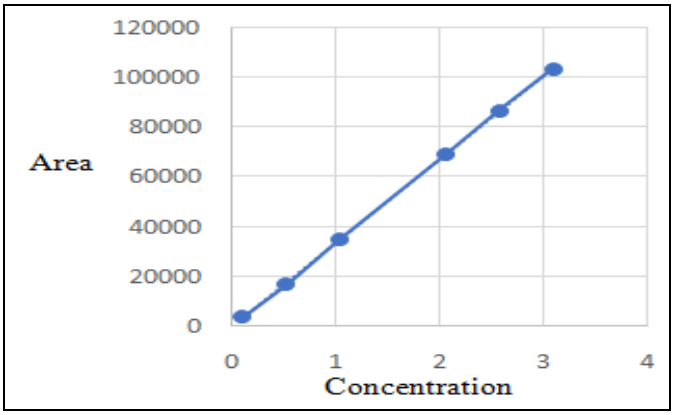

FIG. 15: N-BIS THF DARUNAVIR

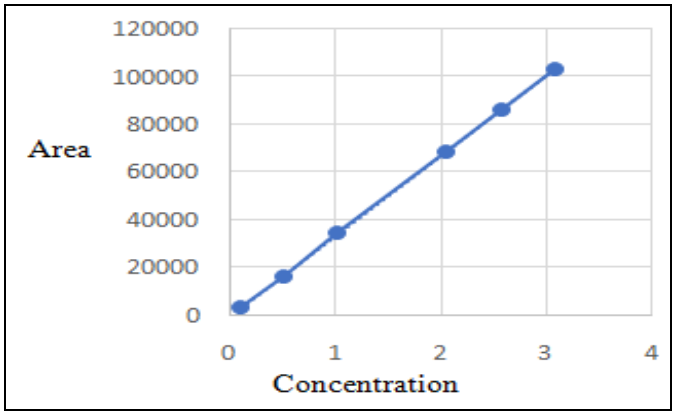

FIG. 16: O-BIS THF DARUNAVIR

CONCLUSION: A simple, Accurate, precise method was developed for the determination of the Darunavir in Tablet dosage form. Retention time of Darunavir were found to be 31.34 min. % RSD of the Darunavir were and found to be 0.2.% Recovery was Obtained as 98.40% Darunavir. LOD, LOQ values were obtained from regression equations of Darunavir were % RSD-3.4, and 0.9 respectively. Regression equation Darunavir is y = 42504.x + 51.43. The method developed was simple and economical that can be adopted in regular Quality control test in Industries.

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