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STABILITY INDICATING DETERMINATION OF DARUNAVIR WITH HPLC IN BLOOD PLASMA SAMPLES

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ABSTRACT

Darunavir is an HIV/ AIDS treatment drug product. Darunavir is used with other medicines line cobicistat or ritonavir. The present objective is to develop and validate the HPLC method for the determination of darunavir in the blood plasma sample. Mobile Phase composed for pH 8.10 buffer (K_2HPO_4), Methanol and acetonitrile in the ratio of 488:162:350 v/v/v. The diluent is a mixture of water and acetonitrile 40:60 v/v. X-Bridge C18 5μm 4.6x250mm column, wavelength 262 nm, injection volume 20μL, flow rate 1.3 ml/min and column oven temperature 35°C were used. Method validation was performed and results were within the acceptable limits. Results confirmed that the developed method is stability indicating and can be applied to analyze the blood plasma samples.

Keywords: Darunavir, blood plasma samples, tablets dosage form, method validation, antiretro viral drug product.

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INTRODUCTION

Darunavir is used as an antiretroviral medication to control HIV / AIDS. This drug is recommended to use with other antiretroviral medicines. Darunavir was innovated by Tibotec at University of Illinois at Chicago[1-2]. It can be used to control after a needle stick injury. Darunavir is approved in the USA in the year of 2006 and it is listed in WHO essential medicines. Darunavir drug is available in Darunavir/ Cobicistat, Darunavir/ Ritonavir combination single pill[3-4]. Darunavir is well tolerated by patients however it has side effects like high blood sugar, diabetes, muscle pain, tenderness, abdominal pain, and constipation, vomiting and headache[5-6]. Darunavir is available in ethanolate form and in the market, it is available in tablets dosage form with 75 mg, 150 mg and 600 mg.

Present research work objective was to develop a simple, stability indicating HPLC method for the quantification of darunavir in blood plasma and urine samples. Two known synthetic impurities were considered for the HPLC method. Chemical structure of darunavir and its impurities were represented in Fig.-1.

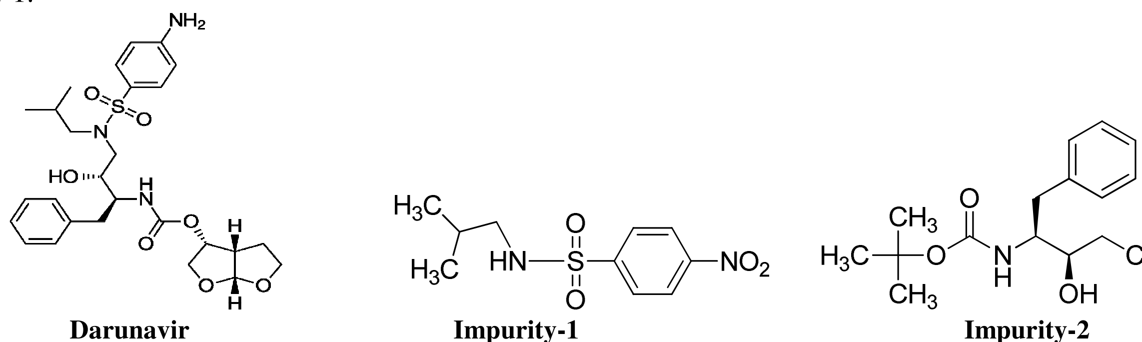

Fig.-1: Chemical Structure of Darunavir and its Impurities

Darunavir: [(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-2-yl]carbamate

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Impurity-1: N-isobutyl-4-nitrobenzenesulfonamide
Impurity-2: Tert-butyl ((2S,3S)-4-chloro-3-hydroxy-1-phenylbutan-2-yl)carbamate
Literature survey was performed and reported methods by HPLC/ MS are, Takahashi M (2007)[7], Patel BN (2012)[8], Reddy BR (2012)[9], Babu GR (2013)[10], Satyanarayana L (2011)[11], Goldwirt L (2007)[12]. FT-IR method by Kogawa AC (2013)[13]. Main objective of this research work was to develop a simple, stability indicating HPLC method for the determination of darunavir in blood plasma and urine samples.

EXPERIMENTAL

Reagents

Di-potassium hydrogen phosphate anhydrous AR grade, Ortho phosphoric Acid AR grade, Acetonitrile HPLC Gradient Grade, Methanol HPLC Gradient Grade, Water for HPLC or equivalent.

Preparation of Dilute Orthophosphoric Acid

Diluted 1 mL of Orthophosphoric acid to 10 mL with water and mixed well.

Preparation of Buffer Solution (pH 3.0)

Weighed and transferred accurately about 4.35 g Di-potassium hydrogen phosphate anhydrous into 1000 mL water, sonicated to dissolve and mixed well. pH adjusted to 3.0 ± 0.05 with dilute Orthophosphoric acid. Mixed well and filtered through a 0.45µ membrane filter.

Preparation of Mobile Phase

4.35 g Dipotassium hydrogen phosphate anhydrous into 1000 mL water, sonicate to dissolve and mix well. Adjust pH to 8.10 ± 0.05 with dilute Orthophosphoric acid. pH 8.10 buffer, Methanol and acetonitrile in the ratio of 488:162:350 v/v/v respectively.

Preparation of Diluent

Prepare a mixture of Water and Acetonitrile in the ratio of 40:60 **v/v** respectively.

Chromatographic Conditions

Column: X-Bridge C18 5µm 4.6x250mm
Wavelength: 262 nm
Injection volume: 20µL
Flow rate: 1.3 ml/min
Column oven temperature: 35°C

Preparation of Diluted Standard Solution

Weighed and transferred accurately 40mg of Darunavir standard into a 100 mL volumetric flask. 70 mL of diluent added and sonicated to dissolve and mix well. 10 mL of this solution pipetted into 100 mL volumetric flask and made up the volume to 50 mL with diluent and mixed well.

Preparation of Sample Solution

Sample powder equivalent to 40 mg of darunavir weighed and transferred to 100 mL volumetric flask. 70 mL diluent added and sonicated for 30 minutes with intermittent shaking. Diluted up to the mark with diluent and mixed well. 10 ml of solution pipetted and diluted to 100 ml with diluent.

Bioanalytical Sample

The liquid-liquid extraction procedure was used to prepare plasma sample. Darunavir tablets 300mg were used; equivalent to 300mg of Darunavir test sample was spiked into 10ml plasma and stored at 2°C for 1 day. Stored spiked solutions were pull-out from the freezer and allowed reach room temperature. A 0.5ml aliquot was transferred to 10.0 mL polypropylene centrifuge tubes. 5.0 mL of ethyl acetate was used to get the two layers and extraction was completed. Centrifuge tubes were kept on vibramax unit for 15 min. Final solutions were centrifuged at 5000 rpm for 5 min at 4°C. 1ml of the supernatant solution was

transferred into polypropylene tubes and evaporated at 40°C. The dried residue was dissolved in 200µL of the mobile phase. Dilution was performed to reach 40ppm concentration. Samples, 20µL by volume, were then injected into the column and analyzed by HPLC on the same day to avoid any degradation. The column temperature oven was maintained at ambient temperature.

RESULTS AND DISCUSSION

Method Development

Literature survey and analytes nature were understood and initial method development were started with phosphate buffer and acetonitrile in the ratio of 60:40; inertsil C18 250mm, 4.6mm; 1.0ml/min flow rate; 20µL injection volume. Impurity-1 and 2 were eluted along with darunavir. Three components UV absorbance was scanned from 200 to 400 nm to select the maximum UV absorbance wavelength. Based on the UV absorbance spectrum for three analytes 267 nm was selected. Figure-2 represented the UV spectrum for darunavir and its impurities. Later we changed the mobile phase ratio with methanol and buffer pH also studied to get the high resolution between darunavir and impurities. Finally, pH8.1 buffer, acetonitrile and methanol mobile phase gave satisfactory results. Final method individual chromatograms were shown in Fig.-3 to 5.

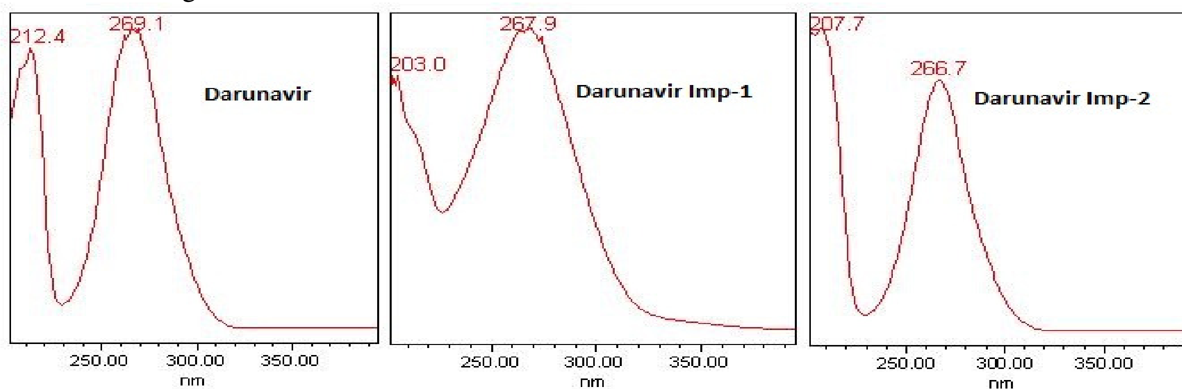

Fig.-2: UV Spectrum Darunavir and its Impurities 1 and 2

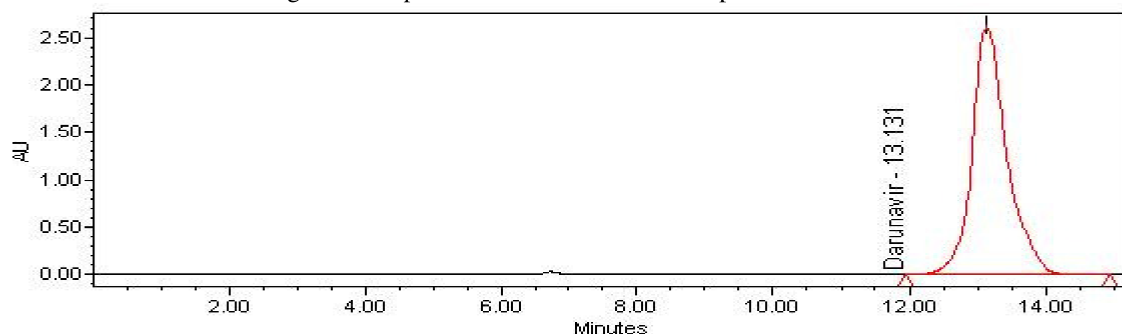

Fig.-3: Darunavir chromatogram

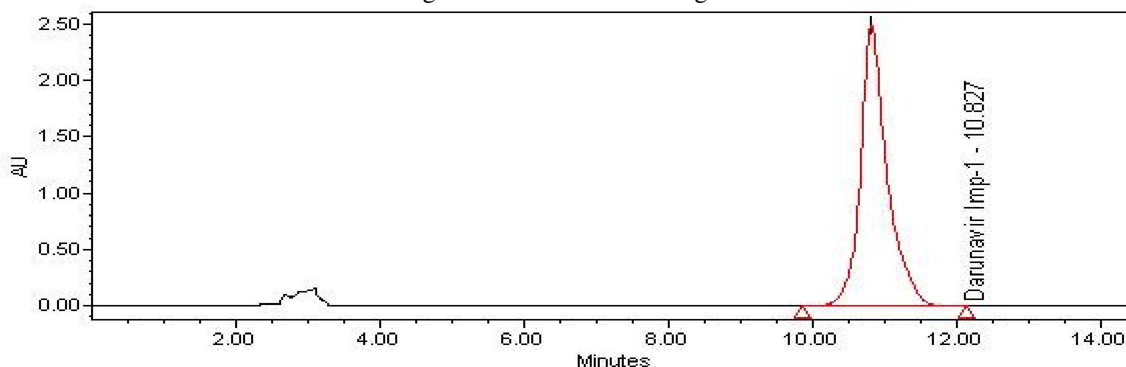

Fig.-4: Darunavir Impurity-1 Chromatogram

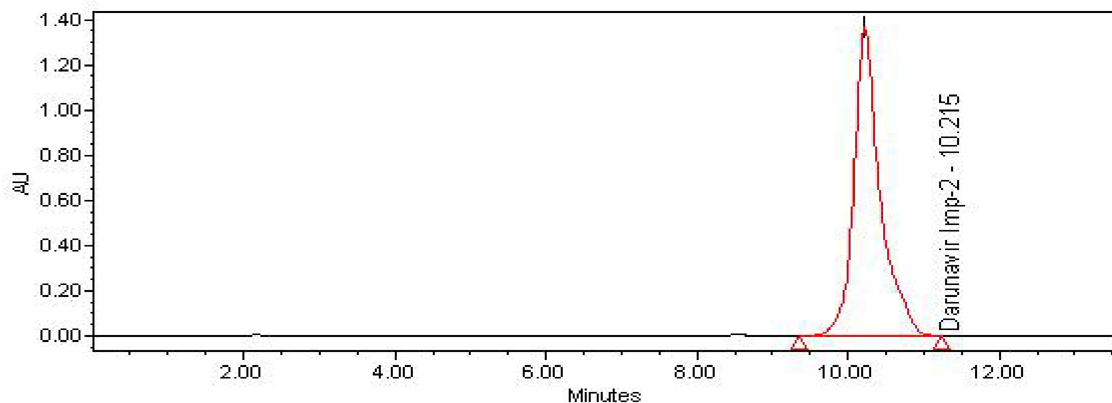

Fig.-5: Darunavir Impurity-2 Chromatogram

System Suitability

Final method was performed to confirm the method suitability and instrument suitability. FigureS-6, 7 and 8 represented the diluent, excipients solution and standard solution 5 injections. Table-1 represents the system suitability results.

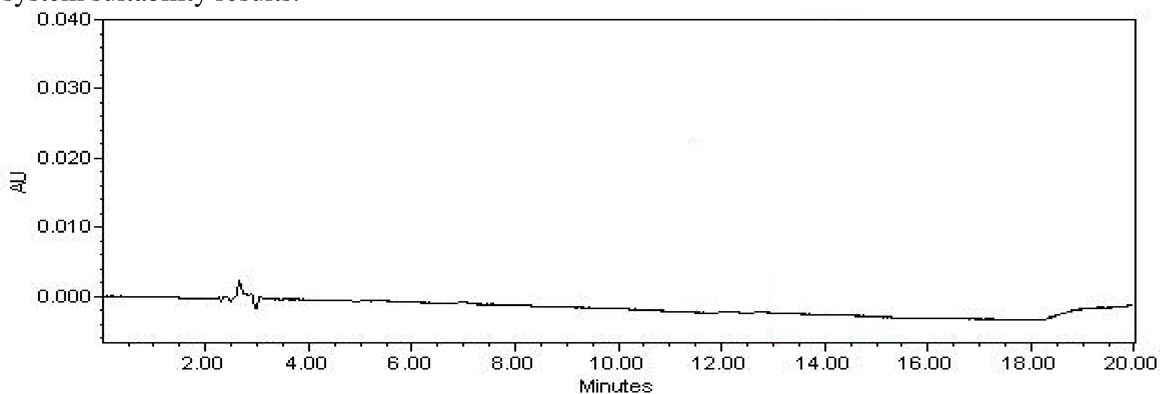

Fig.-6: Diluent Chromatogram

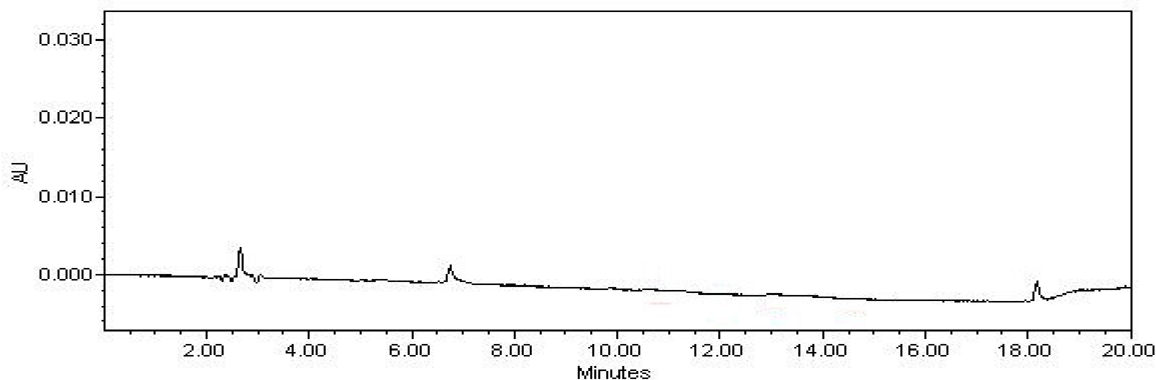

Fig.-7: Excipients Chromatogram

Table-1: System Suitability Results

Injection	Darunavir		
	Retention time (min)	Area	Tailing factor
1.	14.01	1545122	1.18
2.	14.08	1542314	1.15
3.	14.07	1531524	1.14
4.	14.06	1551241	1.16
5.	14.00	1543214	1.17
%RSD	0.25	0.46	NA

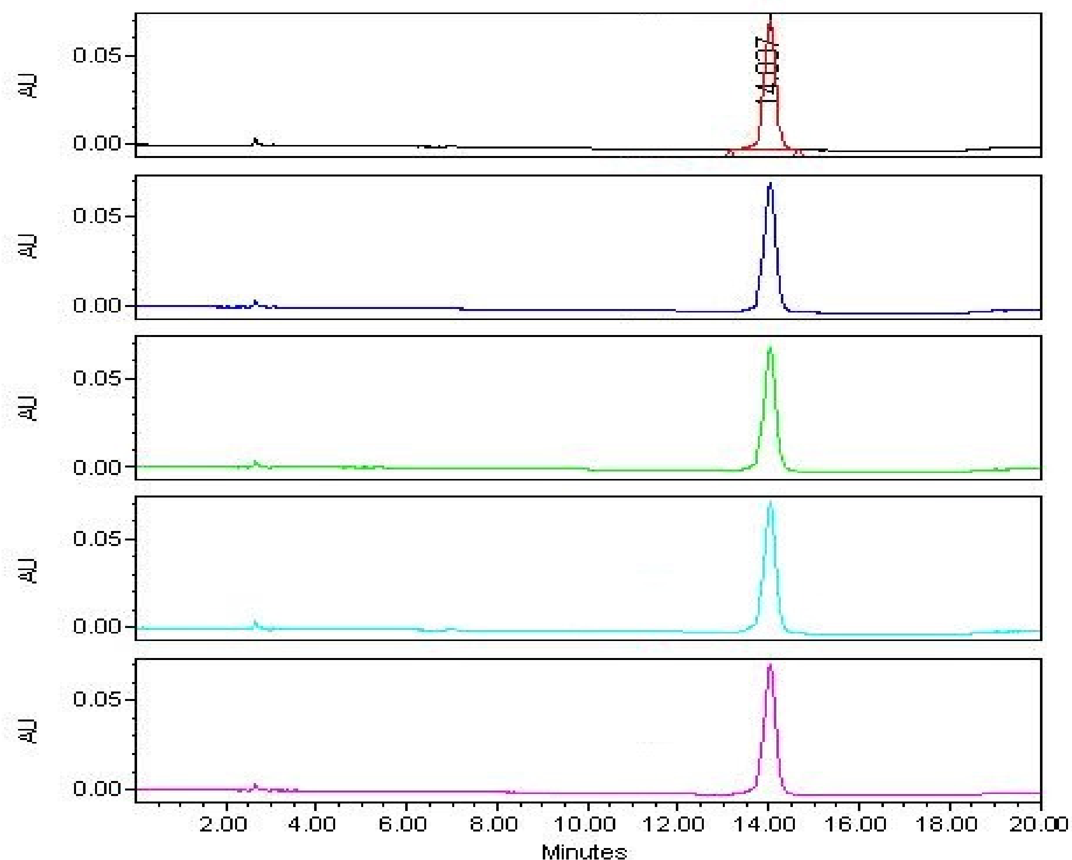

Fig.-8: Five Replicate Standard Solution Injections

Method validation was performed to confirm the method stability indicating property with specificity, precision, accuracy, ruggedness, robustness and linearity.

Specificity

Specificity was evaluated for the interference of blank, placebo and known impurities 1 and 2. Forced degradation studies were performed for acid, base, peroxide, thermal, water and UV light. Blank and placebo and known impurities-1 and 2 were well separated and no interference was observed at the retention time of darunavir. Figure-9 t14 represented the force degradation chromatograms. Table-2 represented the force degradation results and peak purity values.

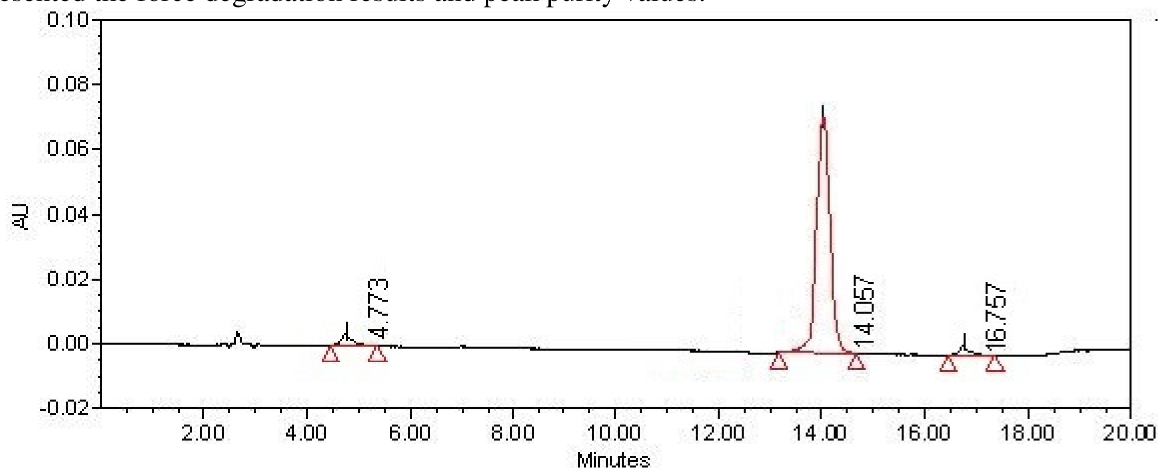

Fig.-9: Acid Degradation Sample Chromatogram

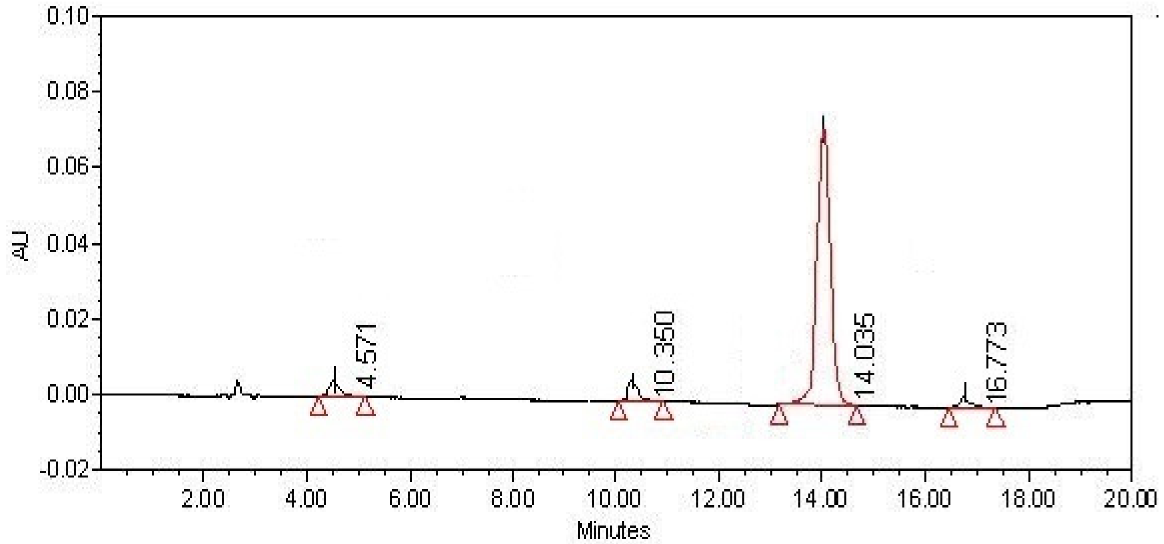

Fig.-10: Base Degradation Sample Chromatogram

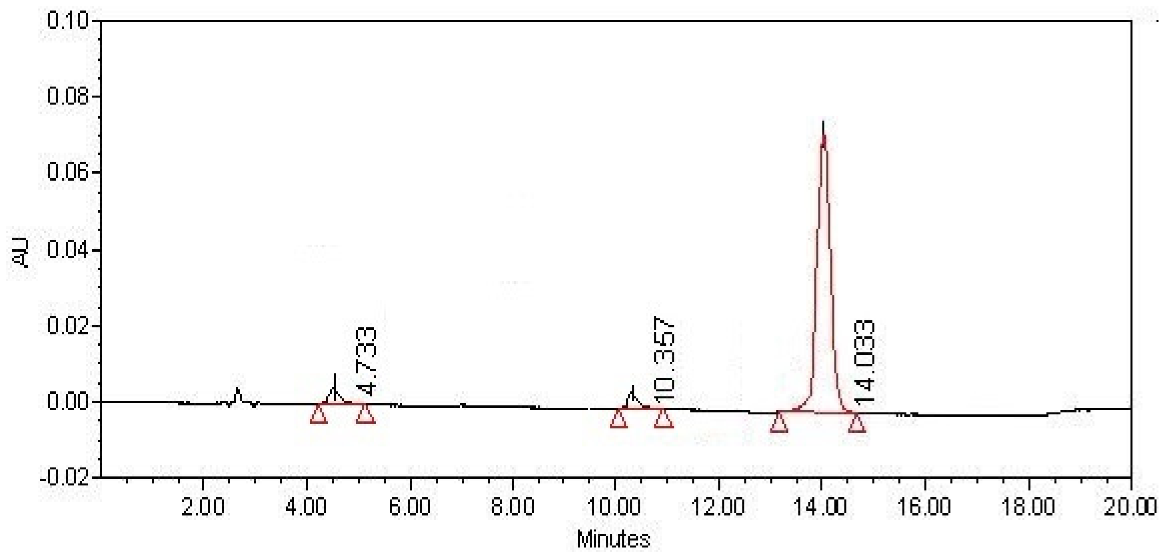

Fig.-11: Peroxide Degradation Sample Chromatogram

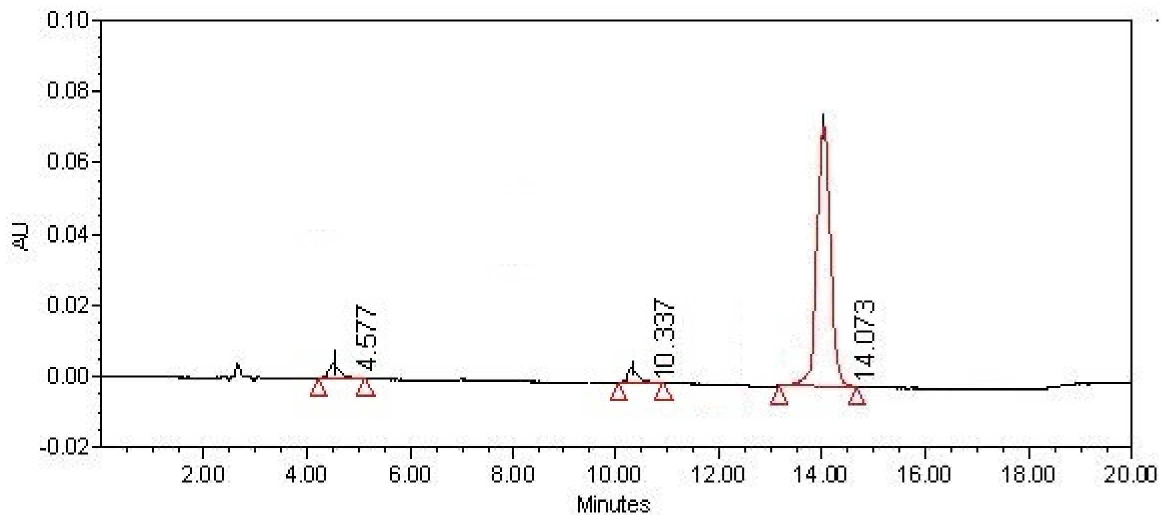

Fig.-12: Thermal Degradation Sample Chromatogram

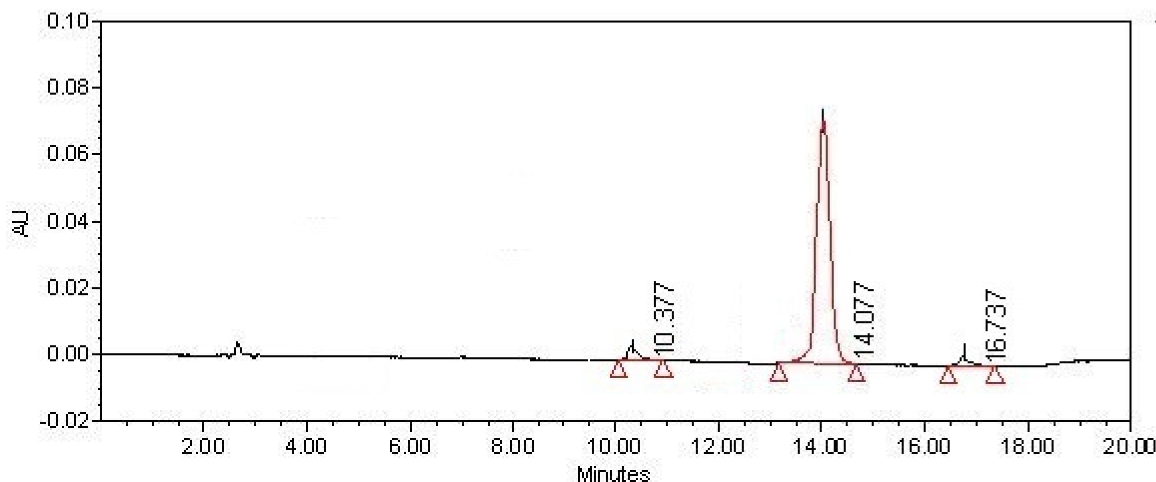

Fig.--13: UV/Visible Degradation Sample Chromatogram

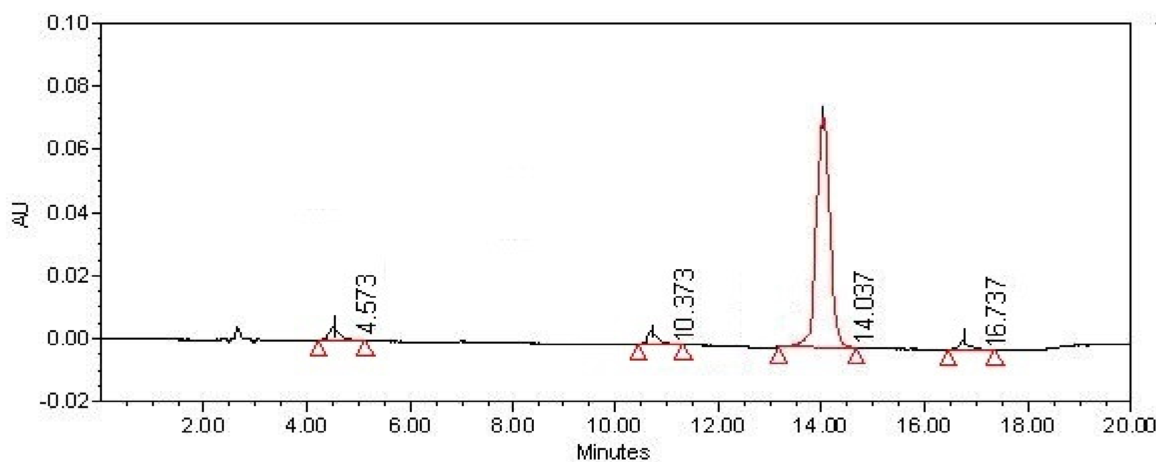

Fig.-14: Water Degradation Sample Chromatogram

Table-2: Stress Study Results

S. No.	Degradation Condition	Darunavir Assay (%)	Purity Angle	Purity Threshold	Purity Flag	Interference
1.	Acid degradation (5N HCl, 1hr, 60°C)	97.04	0.204	0.391	NO	NO
2.	Base degradation (5N NaOH, 1hr, 60°C)	96.98	0.313	0.306	NO	NO
3.	Peroxide degradation (3% H_2O_2, 1hr, RT)	97.89	0.241	0.440	NO	NO
4.	Water degradation (Water, 6hr, 60°C)	96.20	0.288	0.443	NO	NO
5.	Thermal degradation (24hr, 80°C)	97.25	0.276	0.430	NO	NO
6.	UV/ visible light (UV light 200 watt hr/sq. meter) (Visible light 1.2 million lux hrs)	96.80	0.228	0.420	NO	NO

Linearity

Linearity was performed with five different concentration levels with freshly prepared solutions. Concentration levels 20 ppm to 60 ppm was performed. Linearity results were tabulated in below Table-3 and linearity overlay chromatograms were represented in Fig.-15.

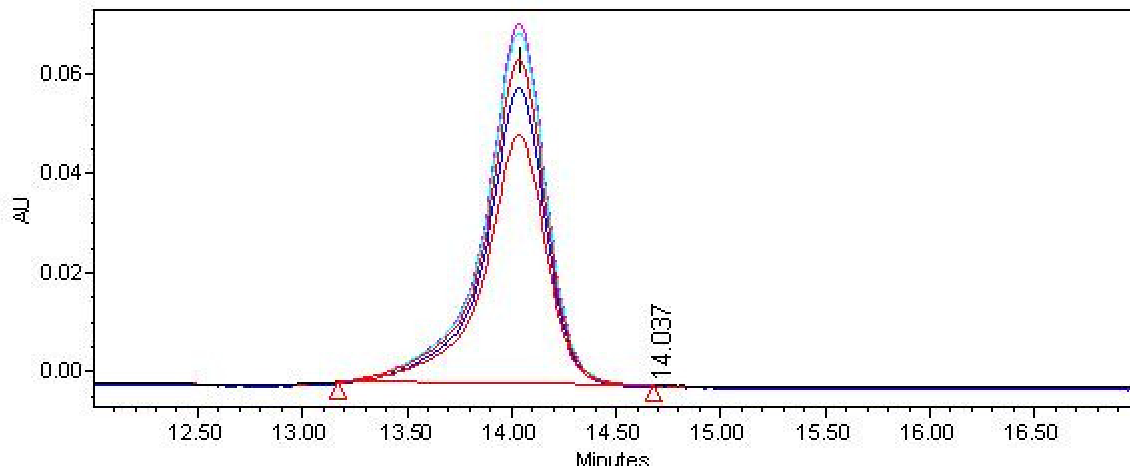

Fig.-15: Linearity Solutions Chromatograms Overlay View.

Table-3: Linearity Results

Linearity Level	1	2	3	4	5	Coefficient of Correlation
Conc. (µg/ml)	20	30	40	50	60	0.9993
Area	652580	113521	1625122	2215401	2681051	

Precision

Method and system precision were performed with 6 replicate test solution preparations. Test solutions were prepared as per the test procedure mentioned in the material and methods section. %RSD and % assay were calculated and reported in table-4. %RSD is within the acceptable limit i.e. 2.0 and % assay values are between 98% and 102%.

Table-4: Precision and Intermediate Precision Results

Precision Results								
S. No.	1	2	3	4	5	6	Average	%RSD
% Assay	99.63	99.67	99.89	99.84	98.99	99.19	99.55	0.33
Intermediate Precision								
% Assay	99.87	99.28	99.59	99.67	99.82	99.68	99.69	0.22

Accuracy

Accuracy was evaluated with 3 different concentration levels. A known concentration of sample was spiked to plasma sample and recovery studies were performed. Below Table-5 represented the accuracy results.

Table-5: Accuracy Results

Darunavir Accuracy													
µg /mL	Added	20.12			40.23						60.31		
	Recov.	20.16	20.61	20.31	40.20	40.15	40.19	40.31	40.29	40.31	60.19	60.21	60.19
% Recovery		100.20	101.34	100.94	99.93	99.80	99.90	100.20	100.15	100.20	99.80	99.83	99.80
% Recovery Mean		100.82			100.02						99.81		

Ruggedness

The ruggedness of the final method was checked with precision test samples. Precision sample-1 and 2 were stored at room temperature and refrigerator. Solutions stability was established for 3 days at room temperature and 5 days for refrigerator conditions. % difference in the assay of darunavir and tailing factor, %RSD was evaluated. Results were tabulated in Table-6.

Table-6: Ruggedness Results

Time in Day	Bench Top Stability Test Solution				Tailing Factor	%RSD	Bench Top Stability Standard Solution
	Test-1	Test-2	Difference				Similarity factor
			Test-1	Test-2			
Initial	99.63	99.67	NA	NA	1.10	0.5	NA
Day-1	99.29	99.35	0.34	0.32	1.16	1.8	1.10
Day-3	99.23	99.20	0.40	0.47	1.13	1.0	1.06
	Refrigerator Stability Test Solution						Refrigerator Stability Standard Solution
Initial	99.63	99.67	NA	NA	1.13	0.5	NA
Day-3	99.59	99.45	0.04	0.22	1.15	1.0	1.05
Day-5	99.29	99.31	0.34	0.36	11	0.2	1.03

Robustness

The ruggedness of the method was determined by verifying the chromatographic conditions such as flow rate, column temperature, mobile phase pH, and acetonitrile and methanol ratio. Tailing factor and %RSD for 5 standard solutions peak area were calculated and result acceptable (tailing factor not more than 2.0 and %RSD is 2.0%). Results were listed in Table-7 Filter verification was performed for PVDF and NYLON filters. Results were tabulated in Table-8.

Table-7: Results of Effect of Variations

Condition	Flow Rate			Column Temperature		
	1.1 ml/min	1.3 ml/min	1.5 ml/min	30°C	35°C	40°C
Tailing Factor	1.1	1.1	1.1	1.1	1.1	1.1
% RSD for 5 Inj.	0.7	1.4	1.2	0.7	0.6	1.4
	Mobile Phase pH			M.P. Acetonitrile Ratio		
	pH 7.9	pH 8.1	pH 8.3	100%	90%	110%
Tailing Factor	1.1	1.1	1.1	1.1	1.1	1.1
% RSD for 5 Inj.	0.7	1.4	1.5	0.7	0.4	1.0
	M.P. Acetonitrile Ratio					
Tailing Factor	100%	1.3	90%	1.6	110%	1.3
% RSD for 5 Inj.		0.6		0.8		0.9

Table-8: Filter Variability Results

	Centrifuged		Nylon Filter				PVDF Filter			
	% Assay		% Assay		% Difference		% Assay		% Difference	
	Spl-1	Spl-2	Spl-1	Spl-2	Spl-1	Spl-2	Spl-1	Spl-2	Spl-1	Spl-2
Darunavir	0.381	0.380	0.377	0.377	0.004	0.003	0.383	0.384	0.002	0.004

CONCLUSION

Darunavir estimation in blood plasma sample was performed using RP-HPLC stability indicating method. HPLC method was developed by using a simple mobile phase with pH 8.1. Mobile phase composed of water,

acetonitrile and methanol (488:162:350 v/v/v). X-Bridge C18 5µm 4.6x250mm column, 262 nm wavelength, 20µL injection volume, 1.3 ml/min flow rate and column heater 35°C were applied. Method validation was performed with precision, accuracy, ruggedness, linearity, specificity and robustness. Results were within the acceptable limits such as tailing factor not more than 2.0, %RSD not more than 2.0%, linearity correlation coefficient 0.999 and % recovery 98% to 102%. The optimized method can be used to determine darunavir in plasma extraction samples.

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