RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF DARUNAVIR IN TABLET DOSAGE FORM

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Article History: Received: 04.05.2023 Revised: 14.06.2023 Accepted: 10.07.2023

Abstract

A simple, economic, sensitive and reliable RP-HPLC method was developed for Darunavir in tablets.From literature survey it was decided to perform gradient mode as other articles were of isocratic mode. Gradient chromatography was analysed on a C18 column with mobile phase (A) as buffer and mobile phase (B) as ACN at a flow rate 1.5 ml/min. The effluent was monitored by UV-detector at 265 nm and overall run time was 15 mins.Calibration curve was linear over the concentration range of 50-150µg/ml. The developed method was validated with respect to ICH guidelines following accuracy,linearity, precision, selectivity and robustness, these parameters examined were in the acceptable limits for analytical method validation.Robustness of the developed method was estimated by making small changes in flow rate (±1ml/min), column temperature(±5%), organic mobile phase ratio (±10%), along with the optimized method.Stability study of solution and force degradation studies was also carried out and found to be satisfactory.The percentage purity of Darunavir was found to be 99.9% and %RSD values for Darunavir were within limit of ≤2.The developed and validated RP-HPLC method was successfully applied for the quantitative estimation of Darunavir in marketed formulation.

Keywords: Darunavir, RP-HPLC, Quantification, Gradient, Validation, ICH guideline

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DOI: 10.31838/ecb/2023.12.s3.633

1. INTRODUCTION

As per World Health Organization (WHO), AIDS epidemic updates, in 2009 new HIV infections were reduced to around 17% by comparing to past eight years. Data from WHO also show that there is huge population affected with HIV compare to early years as people are living longer due to the beneficial effects of antiretroviral therapy along with population growth. WHO concludes that due to the availability of effective treatment in 1996, some 2.9 million lives have been saved.[1]

HIV has continuously been a major global general health issue, having affirmed 36.3 million [27.2–47.8 million] lives so far. There is no treatment for HIV infection. However, with availability to access effective HIV prevention, treatment and care, HIV infection has turned in to become a feasible and preventive persistent health condition, providing people diagnosed with HIV to live long with healthy attribute. It was reported that 37.7 million [30.2–45.1 million] people infected with HIV during the last quarter of year 2020, over 2/3 of (25.4 million) lives in the African Region. In 2020, 680,000 [480,000–1.0 million] people deceased from diseases relatedto HIV and 1.5 million [1.0–2.0 million] people diagnosed HIV.[2]

The human immunodeficiency virus (HIV) assaults the immune system and disables people's defence against many infections and diseases and some categories of cancer that other people with healthy immune systems can easily fight off. As the virus demolishes and damage the function of immune cells, people with infected virus becomes immunodeficient. Immune function is basically estimated by CD4 cell count. .[3]

The molecular weights of darunavir base are 547.73 g/mL, respectively. According to in vitro studies, Darunavir was functional in case of HIV-1 with PI-resistance mutations and against PI-resistant clinical isolates.Darunavir is a formidable HIV-1 PI, chemically similar to amprenavir, and efficient in vitro against viral strains less sensitive to other PIs. DRV belongs to the class of hydroxyethyl amino sulphonamide and is a 2ndgeneration PI developed by the pharmaceutical company Tibotec. DRV was specifically discovered to overcome problems with the older agents in this class, such as indinavir. [4-6]

Darunavir is chemically [(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, it is a HIV protease inhibitor used in the medicament of AIDS and HIV infections. Chemical structure of Darunaviris in Figure 2. Darunaviris an antiretroviral PIwhich is used in the medication and determent of(HIV) infection and the acquired immunodeficiency syndrome (AIDS).Darunavir can lead tomomentary and asymptomatic altitude in serum aminotransferase levels and been related to in frequent prototype of clinically assumed, acute liver injury. In HBV/HCV coinfected individuals, highly efficient antiretroviral treatment with darunavir lead to consequence of an aggravation of the fundamental chronic hepatitis B or C.[7]

HIV is able to escape immunological pressure, to adapt to a category of cell types and growth conditions and to develop resistance against currently available drug therapies. The concludinginvolvesnon-nucleoside reverse transcriptase (NNRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), HIV-protease inhibitors (PIs), integrase inhibitors.[8]

As HIV is a global disease, darunavir is used across the world and therefore stored under different environmental conditions. Because of this, it becomes vital to evaluate the stability of darunavir byfocusing thatpharmaceuticals are especially sensitive to environmental factors.

Figure 1. UV-VIS spectra of Darunavir

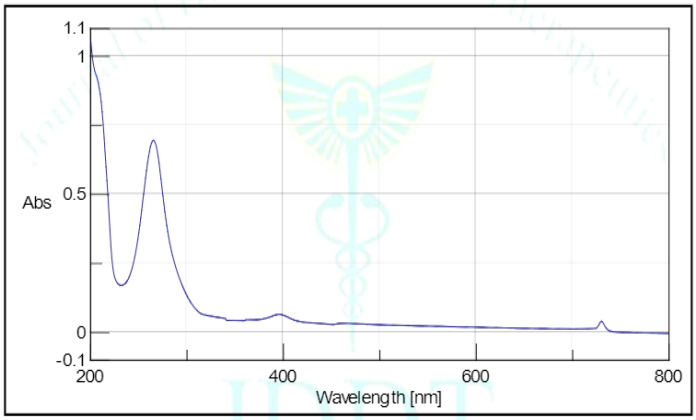
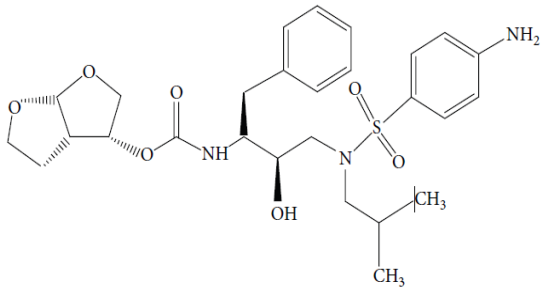


Figure 2. Chemical structure of Darunavir.



Stability as per definition can be defined as 'The capacity of a drug substance or product to withstand the established specifications and to maintain itsquality identity, strength, , and purity throughout expiration period . Stability test of an API or final drug product can provide testimonal on the drug quality and conclude that does the drug get affected by environmental elementsfor eg:temperature and humidity.

Chemical and physical deterioration of drugs may result in variedremedial efficacy and even harmful effects. Therefore, understanding the factors that affects the stability of pharmaceuticals and identifying ways to guarantee their stability becomes vital part . In past few years, the study about the influence of light on the safety of severaldrugs has gained huge scope and importance. Information related to photo stability of drugs can lead to acquire the storage conditions in order to guarantee the safety, efficacy and quality of pharmaceutical products.[9]

Literature survey discloses that various analytical methods have been outline for the evaluation of Darunavir by UV spectroscopy[10], Infrared spectroscopy[11], LC-MS[12], HPLC, HPTLC[13,14]. HPLC methods are performed on Darunavir in isocratic mode but not developed in gradient mode. So purpose of the present study is to carry out a simple, rapid method on Darunavir which is gradient. Therefore, in present investigation a venture has been made to adjudge Darunavir in dosage form using RP-HPLC using a simple mobile phase composition and shorter run time.

2. MATERIAL AND METHOD

An analytically pure sample of Darunavir was obtained as a gift sample from Enaltec Pharma Research, Ambernath, Mumbai, India. Tablet formulation Daruvir (Cipla) were obtained from a local pharmacy market with labelled amount 800 mg per tablet.

Table 1: Chemicals and Reagents

Name	Grade	Manufacturer
Water	Milli-Q	Millipore
Acetonitrile	HPLC Gradient Grade	Rankem
Potassium dihydrogen phosphate	GR Grade	Rankem
Methanol	HPLC Gradient Grade	Rankem
Ortho-phosphoric acid (GR Grade, Merck)	GR grade	Merck

Table 2: Instrument list

Equipment	Make	Model
HPLC	Waters	2695 Alliance Separation Module, (PDA/UV Detector) 2998/2489
Column	ACE	ACE C18, 150 x 4.6 mm, 3µ
pH meter	Thermo Electron Corp.	Orion-4star Orion-3star
Analytical Balance	Mettler Toledo	XS205DU
		AB204-S/FACT

CHROMATOGRAPHIC CONDITIONS

Table 3: Optimized chromatographic condition for Darunavir

Sr.no	Parameter	Description
1	Column	ACE C18, 150 x 4.6 mm, 3µ or equivalent.
2	Flow Rate	1.5 mL/min
3	Injection Volume	5 µL
4	wavelength	265 nm
5	Column Temp	60°C
6	Sample Temp	15°C
7	Run time	15 minutes
8	Retention Time	About 5 minutes for Darunavir
9	Seal wash	Water: Methanol (90:10)
10	Needle wash	Extended needle wash (Methanol: 0.1% Ortho-phosphoric acid (80:20)

Preparation of mobile phase A:
Mobile phase A was made of Buffer Solution pH 4.0.

Preparation of mobile phase B:
Mobile phase Bwas made of Acetonitrile.

Preparation of Buffer Solution pH 4.0:

Weigh accurately 1.36 gm of potassium dihydrogen phosphate in 1000 ml of water and adjust the pH to 4.0 ± 0.05 with Ortho-phosphoric acid and filter through 0.45µ nylon membrane filter.

Diluent 1:
100% Methanol

Diluent 2:

Mobile phase in ratio of 60:40

Preparation ofStandard stock solution:
Weigh and transfer accurately about 80 mg of Darunavir working standard into 50 mL volumetric flask. Add about 30 mL of diluent 1, sonicate to dissolve and dilute up to the mark with diluent and mix. Further dilute 5 mLof this solution to 50 mL with diluent 2 and mix.

Preparation of Sample solution:
Determine the Average weight of 20 tablets. Crush the tablets. Weigh and transfer powder equivalent to 5 tablets in to 500 mL volumetric flask. Add about 350 mL of diluent 1, sonicate for 30 minutes with intermittent shaking, allow it to cool and make up to volume with diluent 1 and mix. Further dilute 4 mL of this solution to 200 mL with diluent 2 and mix. Filter the sample solution through 0.45µ Nylon membrane syringe filter. Discard first few ml of filtrate (160 ppm).

Method validation: The method was validated according to ICH guidelines [21]. The different validation characteristics which were performed are following: Linearity, accuracy, precision, specificity, system suitability, stability of solution, force degradation and robustness.

System suitability parameters: The system suitability parameters were determined by preparing standard solution of Darunavir and the solutionwere injected six times and theparameters like peak tailing, resolution and USP plate count were determined.

Specificity: Blank (Diluent), Placebo should not show any peak at the retention time of Darunavir peak.

Linearity: The linearity of the method is determined by preparing three individual series of solutions.The range of Darunavir is proposed to be from50% to 150%. The obtained peak areas are plotted againstconcentration.

Preparation of linearity solutions:
Preparation of Standard stock solutions: Weigh and transfer accurately about 80 mg of Darunavir working standard into 50mL volumetric flask. Add about 30 mL of diluent 1, sonicate to dissolve and dilute up to the mark with diluent.Further dilute 5mLof this solution to 50 mL with diluent 2 and mixand label it as standard stock solution.From stock solution pipette out 2.5ml, 4 ml, 5ml, 6 ml, 7.5 ml into 50 ml volumetric flak to get 50%, 80%, 100%, 120%, 150% of standard solutions.

Precision
a) Method precision (repeatability): The method precision/ repeatability can be decided by injecting six working standard solutions and six sample injections. Areas of all injection were taken and standard deviation, % relative standard deviation, %assay were calculated.

b)Intermediate precision: The intermediate precision can be determined by injecting six working standard solutions and six sample injections on different days by different operators or by different instruments. Areas of all injection were takenand standard deviation, % relative standard deviation, %assay were calculated. The results acquired were within the acceptance criteria.

Accuracy: Accuracy was determined by recovery study by standard addition method. The known amounts of standard, Darunavir was added to pre-analysed samples from level 50% up to 150% and then injections was carried out through HPLC individually. The results of recovery studies were shown in Table 4.
It was perceived that the mean percentage recovery was found to be for Darunavir which demonstrated that the method was highly accurate.

Stability in Analytical solution:
The Standard and sample solution (Darunavir Film Coated tablets 300 mg) were kept for 24hours at sample temperature condition (15°C) & room temperature (25°C) & were injected time to time continuously to check the solution stability. The data obtained are summarized in Table.

Forced Degradation Studies:

Acid, Alkali, Neutral, Oxidative, and Photo DegradationStudies:
Thestudy of degradation studieswas carried outwith respect to ICH guidelines to stability studies.Samplesolutions were prepared by adding 1N HCl, 1N NaOH, and (30) % H_2O_2; they werestored at room temperature for 24 , 5, and 14 h at 70°C and 50°C in bath. After that, aliquots ofthese solutions were removed and evaluated by LCmethod.These preparations were exposed to UVC radiation (254 nm)for 1 h. The stress degradation was carried out exposing thesolutionsin flasks. Control samples protected from light with aluminum foil were also placed in exposition concurrently inthe light chamber. After that, aliquots of these solutions were removed and evaluated by LC method.

Method robustness: The robustness can be estimated by varying the following parameters: Robustness of thedeveloped method was carried out by making small intended changes in flow rate (±1ml/min), column temperature(±5%), organic mobile phase ratio (±10%), along with the optimized method.

Development and optimization of HPLC method: The current work was intended to develop a RP-HPLC method for the estimation of Darunavir in pharmaceutical dosage form. The solubility of the API was examined in different solvents likemethanol, water, acetonitrile and in different ratios but finally the standard is soluble in acetonitrile and buffer so it was kept as diluent and methanolwas used as second diluent for as a cosolvent.The different mobile phases like acetonitrile and potassium dihydrogen phosphate buffer were used in compositions as gradient with a flow rate of 1.5ml/min.Initially kromosil®"(250mm x 4.6mm x 5µ) and "ODS®" (150mm x 4.6mm x 5µ) columns with different temperatures like 30, 35, 40, 45°C were used but the retention time, run time and peak resolution were not exact and the problem was get rid by using ACE C18 column (150mm x 4.6mm x 3µ) kept at 60°c with a run time of 15 minutes. Finally, the method was optimized by altering the mobile phase composition / ratio and the optimized wavelength of drug was found to be at 265 nm.

System suitability parameters: The system suitability tests were performed before performing the validation.The parameters were within the acceptance criteria like retention times were 5.083 min, plate count wasmore than 2000, peak tailing was less than2 and the %RSD of peak areas of six injections were ≤ 2% (Table 4). Hence the proposed method was effectively applied to routine analysis without any problems.

Specificity: The data demonstrates that the retention time of Darunavir is comparable between Identification solutions, standard and sample solution. There is no interference of Blank, Placebo at the retention time of Darunavir peak.(Table 5).

Linearity range: The linearity range was in the interval of Darunavir (50-150µg/ml) respectively. These were represented by a linear regression equation as follows: y (Darunavir) = 6749.52x + 18,580 (R^2 =1.00) . Regression line was official by least squares method and correlation coefficient (R^2) for Darunavir was found to be greater than 0.999. Hence the curves established were linear. (Table 6).

Precision: Six replicates'injections at the same concentration were examined on same day and 2 different days for confirming the variation in the precision and the % RSD for Darunavir were within acceptable limit of less then2. Hence the present optimized method is reproducible on different days with different analyst and column. This suggest that the method is precise (Table 7 &8).

Accuracy: The percentage recoveries for Darunavirwas found to be 99.2% respectively (Table 8). The results of the recovery studies undoubtedly demonstrate accuracy of the proposed method.

Stability in Analytical solution: The system suitability criteria are fulfilled.The data shows that absolute difference of % assay for Darunavir obtained up to 27 hours at 15°C and 28 hours at 25°C is within ± 2, thus the

sample solution is stable up to 27 hours at 15°C and 28 hours at 25°C.The data shows that cumulative % RSD for Darunavir standard solution up to 27 hours at 15°C as well as 25°C is less than 2.0%, thus the standard solution is stable up to 27 hours at 5°C as well as 25°C.(Table 9-12).

Forced Degradation Studies:
The purity angle is less than purity threshold for Darunavir peak in all conditions. The peak is pure and thus the method is stability indicating with respect to forced degradation studies.(Table 13)

Robustness: Robustness of the proposed method demonstrated a non-significant alteration through analysis of the sample and standard Darunavir solution (Table 9). After this the results acquired were compared with that of optimized method. It was concluded that there is no significant changes in standard deviation, relative standard deviation, theoretical plates, retention time and USP tailing factor by carrying out changes in few parameters.

Assay:
The Content of Darunavir in the pharmaceutical dosage form was found by using the developed method. The percentage purity of Darunavir were found to be 99.9% and %RSD values for Darunavir were within limit of ≤2.

3. CONCLUSION

A new, simple, rapid and precise high performance liquid chromatographic method was developed by gradient mode for the estimation of Darunavir in pharmaceutical dosage form. Hence this method can be applied for the estimation of Darunavir in drug testing laboratories and pharmaceutical industries.

Conflict of Interest:
The authors have no conflicts of interest regarding this investigation**.**

Acknowledgments:
The authors are grateful to Enaltec lab for providing training facilities & free gift samples of Darunavir. The author is indebted to thank Management & Principal of Oriental College of Pharmacy, Navi Mumbai for permitting to avail the needed research facilities, library, and internet facilities & all kind of support.

Table 4: System suitability parameters for Darunavir

	Standard
Symmetry factor	**1.0**
Theoretical Plates	**15254**
Area	1060850
	1056864
	1053905
	1058053
	1051934
Mean	**1056321**
%RSD	**0.3**
Tailing	**1.23**

Table 5: Specificity parameters for Darunavir

Component	Retention time (min)	Ep Theoretical plates	Symmetry factor	Purity angle	Purity threshold
Blank	-	-	-	-	-
Placebo (800mg)	-	-	-	-	-
Standard Solution					
Darunavir	5.083	16401	1.0	0.40	1.73
Sample solutions					
Darunavir (800mg)	5.095	16691	1.0	0.49	1.54

Table 6: Linearity of Darunavir

Level (in %)	Concentration (ppm)	Response			
		Area-1	Area-2	Area-3	Mean
50	79.776	524135	519209	526751	523365
80	127.642	841205	819415	838644	833088
100	159.552	1061179	1073967	1068204	1067783
120	191.463	1274046	1271451	1262301	1269266
150	239.328	1632132	1576780	1585382	1598098
Correlation coefficient (R)					**1.000**
(R^2)					**1.000**
SLOPE					**6749.521**
Y-INTERCEPT					**-18580.985**

Table 7: Method precision

Sample No.	Response			% Assay
	Area-1	Area-2	Mean	
1	1083034	1093417	1088226	102.1
2	1070213	1071134	1070674	100.5
3	1070310	1069164	1069737	100.4
4	1070046	1068106	1069076	100.3
5	1076621	1074234	1075428	100.9
6	1075630	1072219	1073925	100.7
Mean				**100.8**

% RSD	**0.7**

Table 8: Intermediate precision

Sample No.	**Response**			**% Assay**
	Area-1	**Area-2**	**Mean**	
1	1028686	1039310	1033998	101.7
2	1030316	1034649	1032483	101.5
3	1036163	1032858	1034511	101.7
4	1028121	1027083	1027602	101.1
5	1029478	1037396	1033437	101.6
6	1057356	1037979	1047668	103.0
Mean				**101.8**
% RSD				**0.6**

Table 8: Determination of Accuracyof Darunavir

% Level	**Mean response**	**% Recovery**	**% Mean recovery**	**% RSD**
50 %_1	522928	100.0	99.8	0.3
50 %_2	522381	99.9		
50 %_3	520084	99.5		
100 %_1	1040299	99.5	99.2	0.3
100 %_2	1034993	99.0		
100 %_3	1034806	99.0		
150 %_1	1558751	99.4	100.3	1.0
150 %_2	1589187	101.3		
150 %_3	1569978	100.1		

Table 9: Robustness data for Darunavir.

Sr.no	**Condition**	**%RSD of Darunavir**	**% Assay**
1	Flow rate (-) 1.4 ml/min	0.5	99.5
2	Flow rate (+) 1.6 ml/min	0.4	99.7
3	Mobile phase (-) IOC	0.3	100.8

4	Mobile phase (+) DOC	0.8	99.3
5	Temperature (-) 55°C	0.6	101.6
6	Temperature (+) 65°C	0.7	98.4
7	PH (3.8)	0.8	100.0
8	PH (4.2)	0.6	98.8
9	Wavelength 260	0.3	101.7
10	Wavelength 270	0.4	101.8

Table 9: Stability in analytical solution for Standard solution (15°C)

Solution Stability Standard		
Time	**Standard area**	**Cumulative % RSD for Standard**
Initial	988666	NA
	989177	
	1014978	
	1003791	
	992334	
13HRS	1006991	1.1
22 HRS	968751	1.5
27 HRS	995670	1.4

Table 10: Stability in analytical Standard solution (25°C)

Solution Stability Standard		
Time	**Standard area**	**Cumulative % RSD for Standard**
Initial	988666	NA
	989177	
	1014978	
	1003791	
	992334	
14 HRS	993466	1.0
22 HRS	987251	1.0
27 HRS	1000622	1.0

Table 11: Stability in analytical Sample solution (15°C)

Solution Stability Sample		
Time	**% Assay**	**Absolute % Difference**
Initial	98.5	NA
14 HRS	99.4	-0.90
22 HRS	98.1	0.40
27 HRS	100.2	-1.70

Table 12: Stability in analytical Sample solution (25°C)

Solution Stability Sample		
Time	**% Assay**	**Absolute % Difference**
Initial	98.5	NA
15 HRS	99.4	-0.90
23 HRS	99.0	-0.50
28 HRS	97.6	0.90

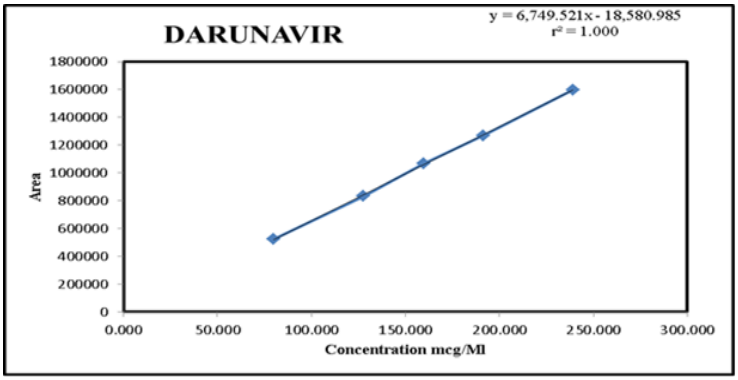


Figure 2: Linearity of Darunavir

Table 13: Forced degradation

Conditions	**% Assay Darunavir**	**Purity angle**	**Purity threshold**
Control	100.6	0.493	1.323
Acid degradation (1 N HCL)	86.4	0.503	1.445
Base degradation (1 N NaOH)	88.4	0.484	1.389
Peroxide degradation (30% H2O2) 70°C 5 hrs	102.0	0.475	1.322
Heat (50°C) 24 hrs	102.7	0.502	1.331
Photolytic study			
Amber flask +Aluminium Covered (Control)	102.0	0.496	1.351
Amber flask	101.8	0.494	1.351

Clear glass	101.8	0.484	1.311

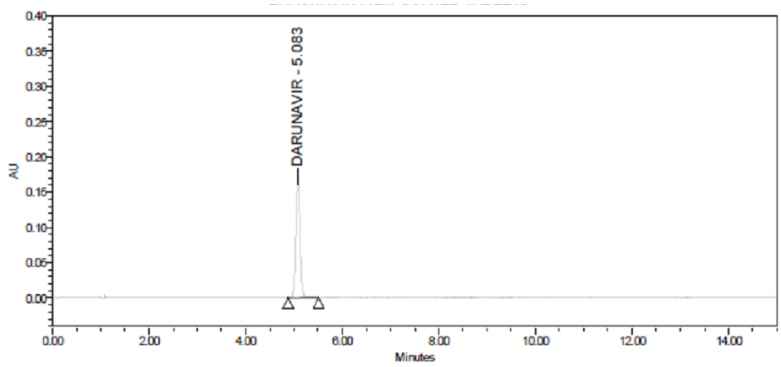


Figure 3: Chromatogram of Standard solution

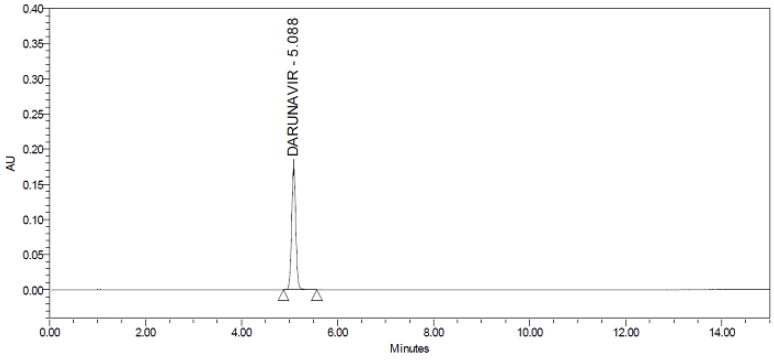


Figure 4: Chromatogram of Sample solution

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