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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF DARUNAVIR- AN ANTIRETROVIRAL AGENT BY RP- HPLC METHOD

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ABSTRACT:
A simple and precise HPLC method was developed for estimation of Darunavir in pure drug and pharmaceutical dosage form. The separation was carried out using ACE C18, 150 x
4.6 mm, 3.0 µm column, mobile phase ACN: Buffer pH 4.0 in the ratio of 40:60 v/v, pH = 4 with the flow rate of 1.5 ml/min using detector MD-2010 plus at 265 wavelength. The
Retention time was found to be 4.951. Calibration curve was linear with correlation coefficient of 0.999 over concentration range of 20-100 µg/mL for Darunavir. Method was foundto be reproducible with relative standard deviation (RSD) for intra and inter day precision less than 2%, the method was validated by evaluation of different parameters such as System suitability, Specificity (Identification, Interference & Peak Purity), Linearity, Accuracy, Precision, Limit of Detection and Limit of Quantitation, Stability in analytical solution & Robustness. Literature survey reveals that there are very few HPLC methods were available. Hence an attempt has been made to develop an RP-HPLC method for the assay method of darunavir. The developed method was validated for System Suitability, Specificity, Linearity, Accuracy, Precision, Limit of Detection and Limit of Quantitation, Solution Stability and Robustness.

Keywords: Darunavir, RP-HPLC, Method Development, Validation.

1. INTRODUCTION:

Darunavir was designed to form robust interactions with the protease enzyme from many strains of HIV, including strains from treatment-experienced patients with multiple resistance mutations to PIs. Darunavir exists as polymorphs in form of Darunavir amorphous, Solvates and Hydrates. The ethanolate and hydrate forms exist in the form of crystals, whereas the non-solvated form is amorphous. When in the formed crystalline forms exist solvent molecules, water, salt, excipient or impurity has a pseudo polymorphic behavior and it can occur during handling, processing and storing. The tendency of a molecule to form solvates is related to the molecular structure, standards of hydrogen bonding and crystal packing. The

solvent serves to stabilize the structure and desolvation process results in the formation of an amorphous form.

Literature survey reveals that HPLC, LC-MS and IR methods are reported for determination of Darunavir as ethanolate form but no methods are reported for determination of darunavir in amorphous form. Hence it was thought worthwhile.

Darunavir, (3 - [(4 – amino - benzenesulfonyl) - isobutylamino] – 1 – benzyl – 2 - hydroxypropyl) - carbamic acid hexahydrofuro - [2, 3 - b] furan-3-yl ester, is a synthetic nonpeptidic PI developed in 1998 by the pharmaceutical company, Tibotec. The compound was licensed in June 2006 in the United States and in February 2007 in the European Union [1]. Darunavir has been selected as an active pharmaceutical ingredient that is classified as a Class 2 BCS substance and exists in two commercially available forms: crystalline ethanolate and amorphous[39]. Darunavir ethanolate is off-white powder with poor aqueous solubility (solubility of approximately 0.13 mg per mL in water at 25 ◦C) [2]. Darunavir is a non-peptidyl small molecule inhibitor of the protease of HIV-1. The drug inhibits the dimerization of the protease, thus inhibiting it [3]. The protease encoded by HIV-1 is required for the processing of the viral polyproteins encoded by the gag and pol genes into mature virion protein and is therefore a target for antiretroviral therapy for HIV [4].

HIV-PIs (HIV protease inhibitors) have proved to be of great benefit for the millions of people suffering from AIDS [5]. Highly active antiretroviral therapy (HAART) is recognized as the most effective treatment method for AIDS, and protease inhibitors play a very important role in HAART. The combination therapy of HIV protease inhibitors, reverse transcriptase inhibitors, and/or an integrase inhibitor, referred to as highly active antiretroviral therapy (HAART), is the current most effective AIDS therapy [6]. Compared with early-generation PIs, boosted darunavir has a high genetic barrier to resistance and is active against multidrug-resistant HIV isolates. In clinical trials in treatment-experienced patients with HIV-1 infection receiving an optimized background regimen (OBR) [36] twice-daily boosted darunavir was more effective than investigator-selected ritonavir-boosted control PIs (CPIs) or ritonavir-boosted lopinavir [7]. Darunavir inhibits HIV virion maturation by targeting protease. As such, it inhibits the cleavage of HIV gag-pol polyproteins, thereby preventing further infection by inhibiting virus maturation and infection of CD4+ T cells [8].

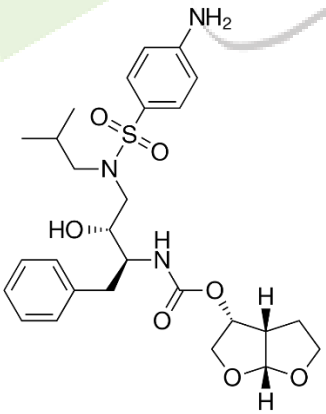

Fig. 1: structure of Darunavir[38]

2. Materials & Methods:

2.1 Chemicals & Reagents:

Chemicals used in the research work are distilled water, Acetonitrile of HPLC grade, Methanol of HPLC grade, Hydrochloric acid of GR grade, Acetic Acid of GR grade, Methanol of GR grade, Potassium Di-hydrogen Phosphate of GR grade, OPA of GR grade, hydrogen Peroxide of GR grade, and Sodium Hydroxide of GR grade

2.2 INSTRUMENTS:

High Performance Liquid Chromatographic Systems:

Jasco LC-Net II/ADC equipped with Quaternary Gradient pump PU-2089 plus, multi-wavelength detector MD-2010 plus with manual injector with 20µL loop and a reserved phase ACE C_{18} column (150x4.6 mm,5µ) with pore size of 100A^{o} was used for chromatographic studies, **UV-visible spectrophotometers:** Jasco v-630**, pH meter:** EI, Model No. 1102012**, Stability Chamber:** THERMOLAB, Model No.: TS 00002008**, Filter:** Membrane filters with cellulose filter paper of 0.45µ**, Sonicator:** PCI Mumbai, Model No. 3.5L 100H**, Weighing balance**: Shimadzu AUX220 &

Calibrated glassware's were used throughout experimental work.

3. EXPERIMENTAL WORK:

3.1 Identification of Raw Material

By UV Spectroscopy:

Between 400 and 200 nm, a standard darunavir solution at 10 µg/ml was scanned in a 1.0 cm cell against a solvent (methanol) blank, and the spectrum of wavelengths were captured.[101]

Fig 1.1 depicts the spectrum of Darunavir standard solution.

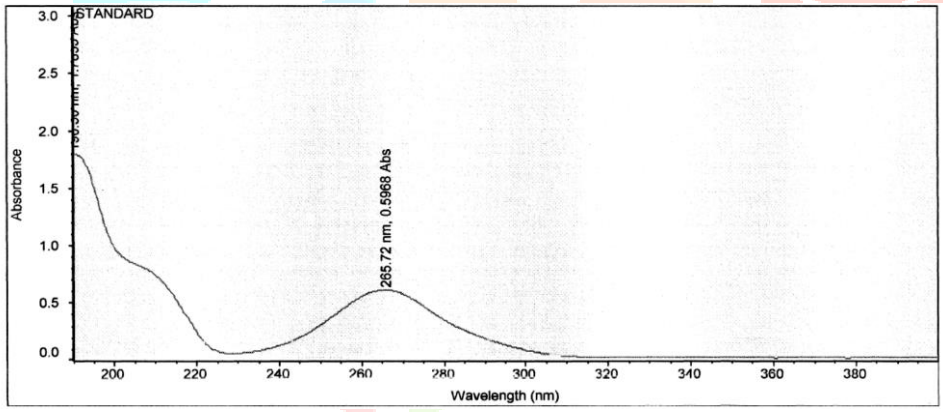

Fig. 3.1 UV spectrum of Darunavir Standard Solution

Table 3.1: UV Wavelength of Darunavir

Reported value (λmax)	**Observed value** (λmax)
266 nm	265.7 nm

From the observed result, the λmax of Darunavir was discovered to be 265.7 nm, which is equal to Standard or Reported value, also indicate no impurities in the Darunavir standard .Hence, was chosen as the detecting wavelength for future investigation.

3.2 Preparation of solutions:

- **Diluent :**100% Methanol
- **Buffer Solution (pH 4.0) preparation:**

  1.36 g of potassium di-hydrogen phosphate correctly weighed dissolved in 1000 mL of water, pH-adjusted to 4.0 ±0.05 using acetic acid, and screened and filter.
- **Mobile Phase preparation:**

  A mixture consisting of Buffer pH 4.0 and ACN in the proportion of 60:40 v/v was prepared. Mixed and sonicated to degas.
- **Darunavir Standard Solution preparation:**

  A precisely measured amount of about 8.0 mg of Darunavir standard was taken in a 50.0 mL conical flask, Diluted up to the mark with the diluent. Sonicated to dissolve and mixed. (Darunavir concentration: 160 µg/mL).
- **DAR-I Impurity Stock preparation:**

  A precisely measured amount of approximately 3.2 mg of DAR-I impurity was taken in a 20.0 mL conical flask; volume was marked up with the diluent. The solution was then sonicated to dissolve and shaken to mix. (DAR-I impurity concentration: 160 µg/mL)
- **Identification solution of DAR-I impurity:**

  In a conical flask, 0.5 mL of Dar-I impurity solution in stock had been diluted to 50.0 mL with diluent and stirred. (DAR-I impurity concentration: 1.6µg/mL).

4. Optimization of chromatographic conditions:

Different Trails were taken for development of the Assay method of Darunavir out of which final trial is given below.

Final Trail:

Chromatographic conditions:

Column : ACE C18, 150 x 4.6 mm, 5.0 µm.

Injection Volume : 20 µL

Flow Rate : 1.5 mL/min

Detector : 265 nm

Sample Temp : 10°C

Column Temp : 65°C

Procedure:

A 20 µL solution of blank, standard darunavir, darunavir spiked solution, Dar-I impurity were injected separately and chromatograms recorded is shown in Fig. 4.1 (a- d) and the observation recorded is shown in table 4.1.

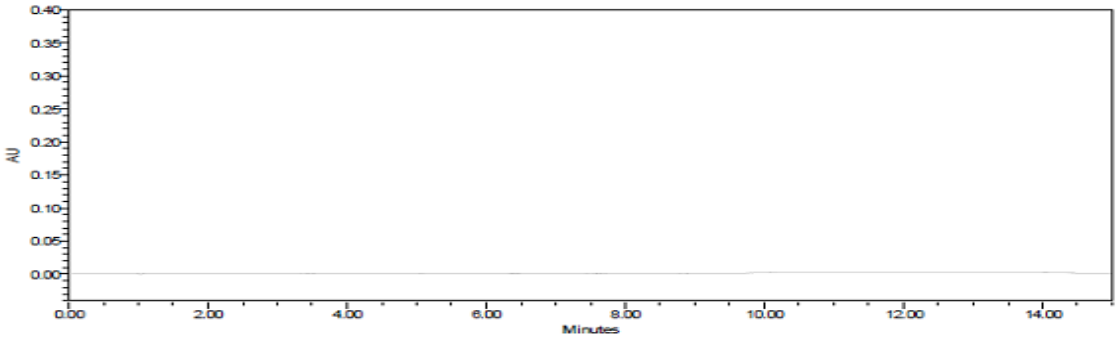

Fig. 4.1 a): Chromatogram of Darunavir Blank Solution

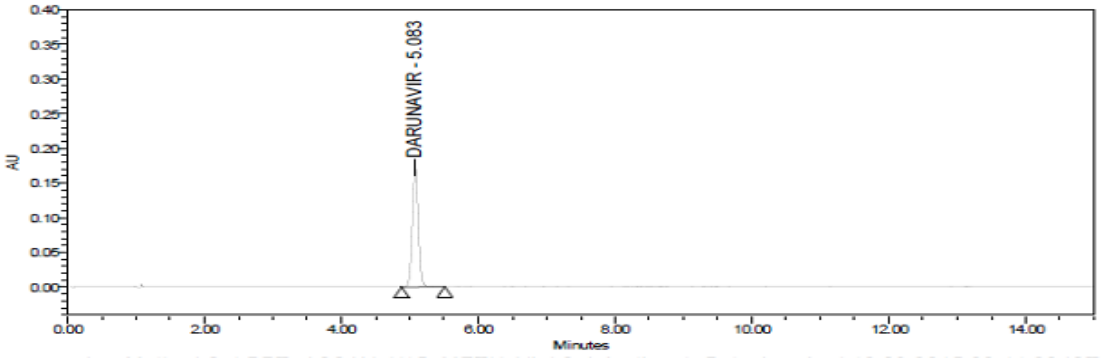

Fig. 4.1 b): Chromatogram of Darunavir Standard Solution

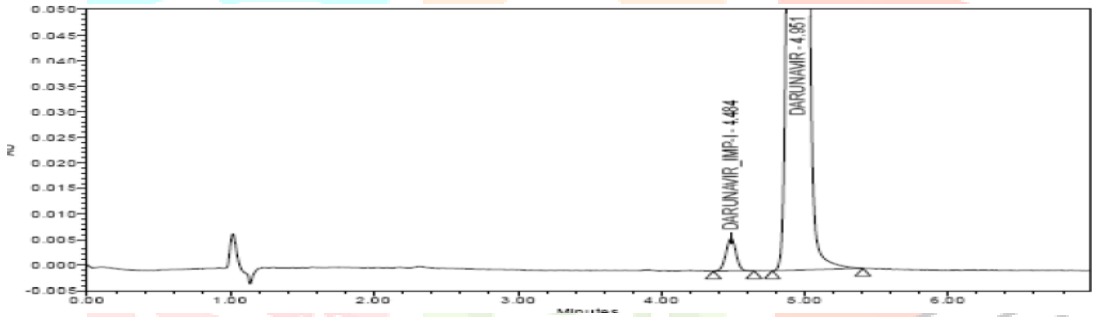

Fig. 4.1 c): Chromatogram of Spiked Darunavir Standard with DAR Impurity –I

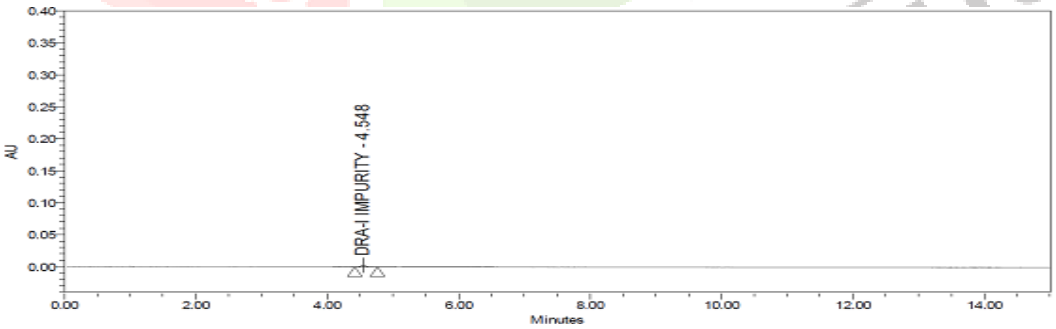

Fig. 4.1 d): Chromatogram of DAR Impurity –I

Table 4.1: Observation of Trial -5

Peak name	Retention Time	Area (mAU)	Theoretical plate	Asymmetry	Resolution
Dar-I impurity	4.484	31048	20567	0.99	-
Darunavir	4.951	4407637	20692	1.01	3.56

Remarks:

The retention time for Darunavir found to be satisfactory. The resolution between Dar-I impurity and Darunavir was found to be 3.56.

Conclusion:

Under above chromatographic condition the resolution, theoretical plate, symmetry was found to be satisfactory, hence selected and finalized for further experimentation.

The final chromatographic conditions are shown in Table 4.2.

CHROMATOGRAPHIC CONDITION:

Table 4.2: Final Chromatographic Conditions

Parameters	**Condition**
Stationary Phase	ACE C18, 3µ (150 × 4.6 mm)
Mobile Phase	ACN : Buffer pH 4.0 (40:60v/v)
Flow Rate	1.5 mL/min
Detection Wavelength	265 nm
Pump Mode	Isocratic
Injection Volume	20 µL
ColumnTemperature	58-60°C
SampleTemperature	15°C

5. METHOD VALIDATION (RESULT & DISCUSSION)

5.1. System suitability:

- **Study of System Suitability Parameters:**

Table 5.1: Results of System Suitability Parameters

Sr. No.	**Weight of drug (mg)**	**Response Area(mAU)**
1	80.12	1060850
2		1056864
3		1053905
4		1058053
5		1051934
	Mean	1056321
	%RSD	0.3
	Symmetryfactor	1.0
	TheoreticalPlates	15254
	Retention Time	5.083

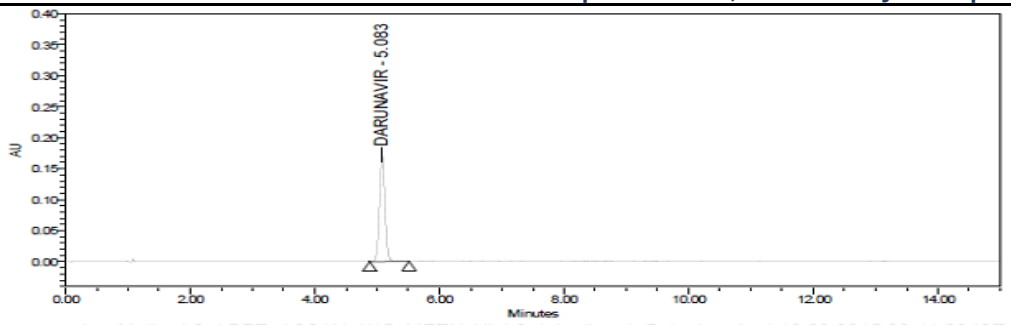

Fig. 5.1: Chromatogram of Darunavir System suitability Solution

From the chromatogram the retention time of Darunavir was found to be 5.083. Five replicate injections of the reference solution showed a relative standard deviation of 0.3. The system is acceptable for analysis in accordance with the acceptance criteria (value shouldn't be greater than 2).

5.2. Specificity:

Table 5.2: Observation of Specificity

(Identification and Interference, Peak purity)

Component	Retention Time (min)	Theoretical Plates	Symmetry Factor	Purity Angle	Purity Threshold
Blank					
Blank	-	-	-	-	-
Standard Solution					
Darunavir	5.083	16401	1.0	0.40	1.73
Identification solutions					
DAR-I	4.548	16124	1.1	5.68	9.83
Darunavir - Spiked Sample					
Darunavir	5.095	16691	1.0	0.49	1.54

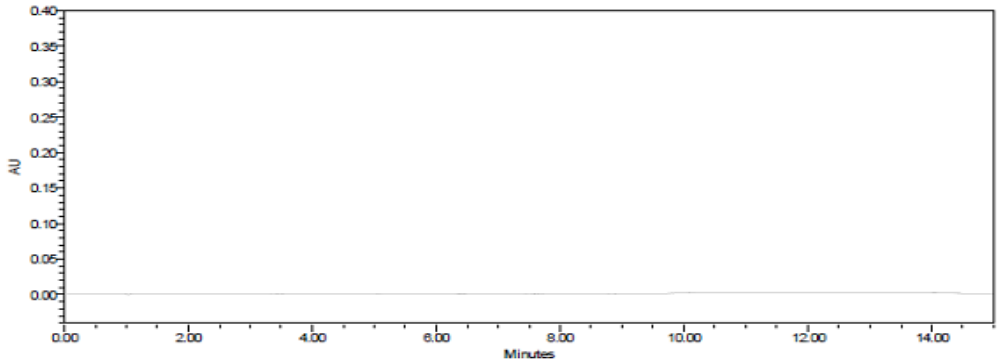

Fig. 5.2 a): Chromatogram of Blank

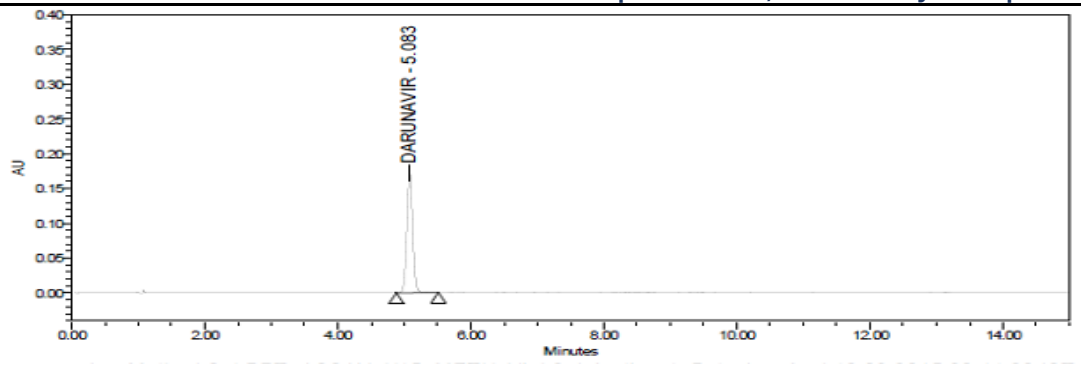

Fig. 5.2 b): Chromatogram of Darunavir Standard Solution

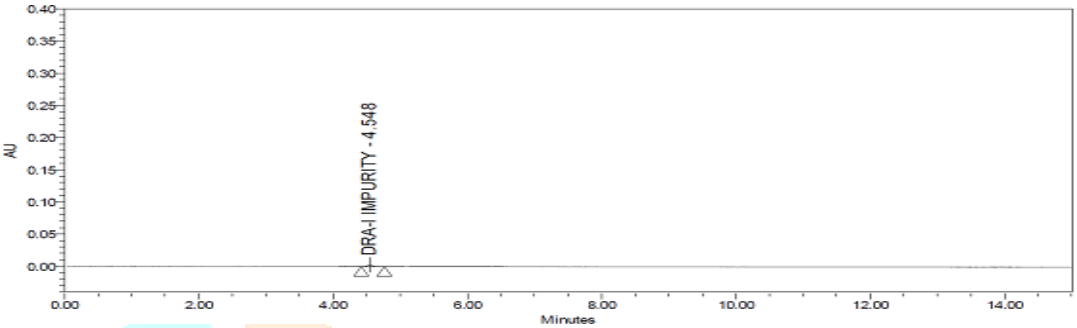

Fig. 5.2 c): Chromatogram of DAR Impurity –I

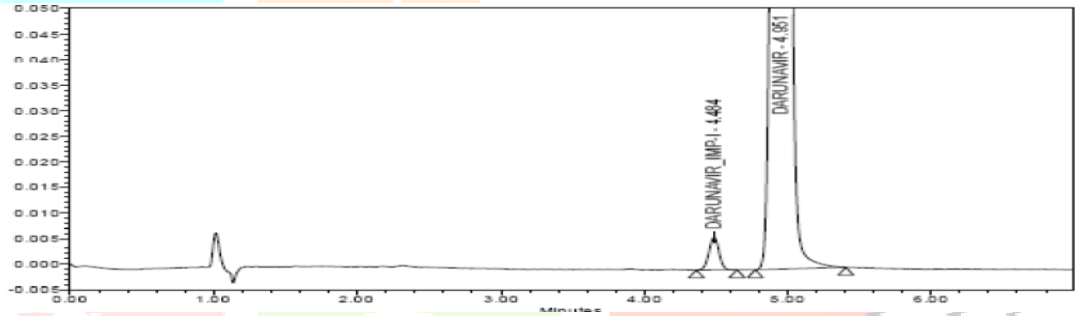

Fig. 5.2 d): Chromatogram of Spiked Darunavir Standard with DAR Impurity –I

There were no interference peaks observed between the blank, standard and the impurity solution as indicated by purity plot and purity factor determination in Table 5.2.

5.3. Linearity and Range:

Table 5.3: Observation of Linearity

	Response Peak Area (mAU)			
% Level	**Replica 1**	**Replica 2**	**Replica 3**	**Mean**
20	420132	410184	410112	413476
40	810264	821102	819521	816962
60	1220296	1210564	1218546	1216469
80	1620528	1642547	1654782	1639286
100	2015066	2029571	2108561	2051066
Correlation coefficient (R)				0.999
(r2)				0.999
Slope				12805
% Y-intercept				3.21

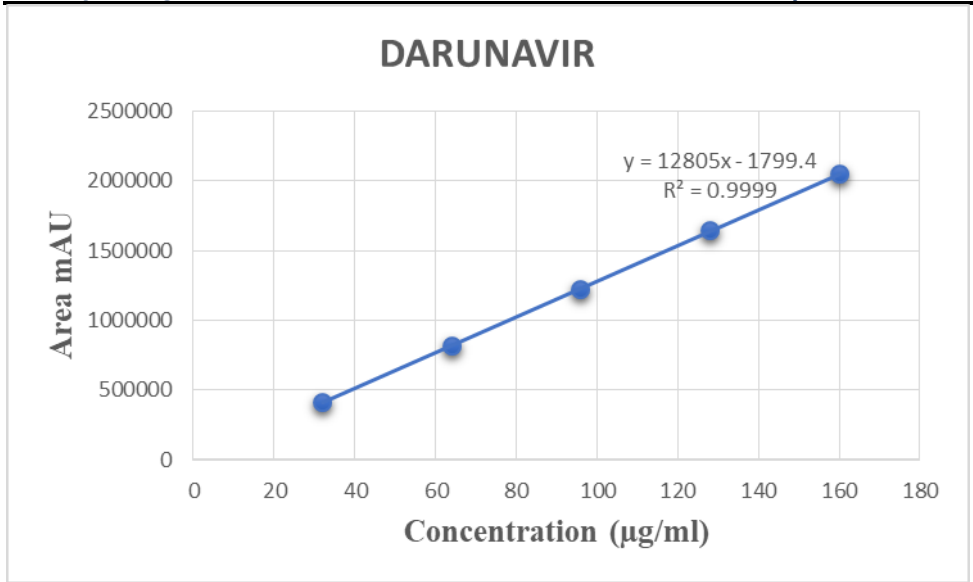

Fig. 5.3: Plot of Calibration curve of Darunavir

The observations are shown in Table 5.3. From the data plot by spiked level concentration Vs area was constructed for the Darunavir. The correlation coefficient of Darunavir was found to be 0.99, and graphical plots are shown in Fig. 5.3. Hence, this indicates that the proposed method is Linear.

5.4. Accuracy (Recovery):

Table 5.4: % Recovery for Darunavir

Sr.no	Level (%)	Amount Added (mg)	Peak Response Area (mAU)	Amount Recovered	% Recovery
1	50%	40.1	158410	400.79	99.89
		40.2	158356	400.38	99.57
		39.9	158126	398.64	99.71
2	100%	79.9	210156	793.04	99.15
		79.9	210589	796.32	99.61
		80	210659	796.86	99.54
3	150%	120.7	262358	1188.75	99.06
		120.1	262589	1190.35	99.03
		120.2	262781	1191.35	99.12
Mean					99.4
±SD					0.32
%RSD					0.32

On the basis of recovery studies carried out using the traditional addition approach, the proposed method's accuracy was determined. The recovery study of Darunavir was observed to be very close to 100% representing the accuracy of the drug and also shows that excipients do not interfere in the estimation. The results and statistical data are shown in Table 5.4**.** According to acceptance criteria mean recovery should be in the range of 98.0% - 102.0% and found to within the range, hence the method was found to be accurate.

5.5. Precision:

5.5.1. System Precision:

Table 5.5 (a): Observation for System precision

Injection	Area (mAU)
1	1060850
2	1056864
3	1053905
4	1058053
5	1051934
6	1054304
Mean	**1055985**
SD ±	**3233.582**
%RSD	**0.3**

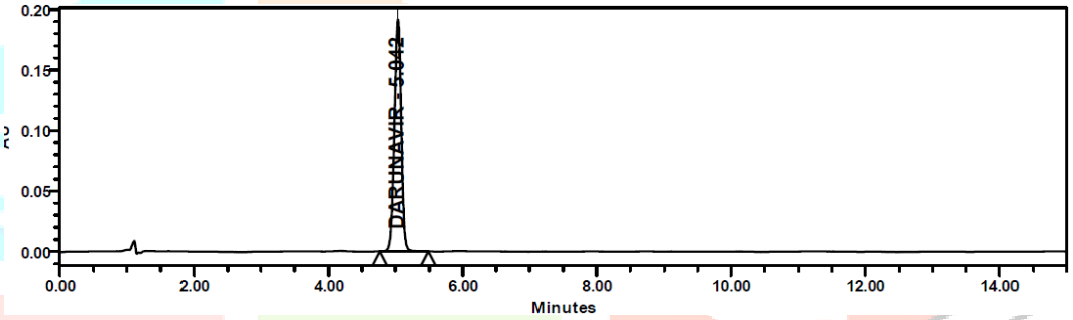

Fig. 5.5 (a): Chromatogram of Darunavir Standard Solution

5.5.2 Method Precision:

Table 5.5 (b): Observation for Method precision

Sample No.	Area (mAU)			% Assay
	Replicate-1	Replicate-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**

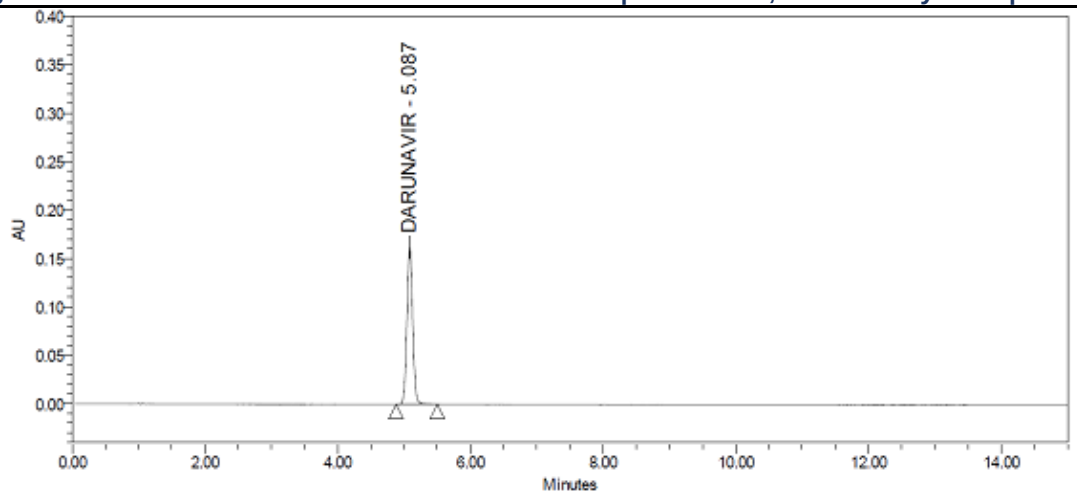

Fig. 5.5 (b): Chromatogram of Darunavir sample solution

5.5.3 Intermediate Precision (Ruggedness):

Table 5.5 (c): Observation for Intermediate precision

Spl. No.	Area			% Assay
	Replica-1	Replica-2	Mean Area	
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**

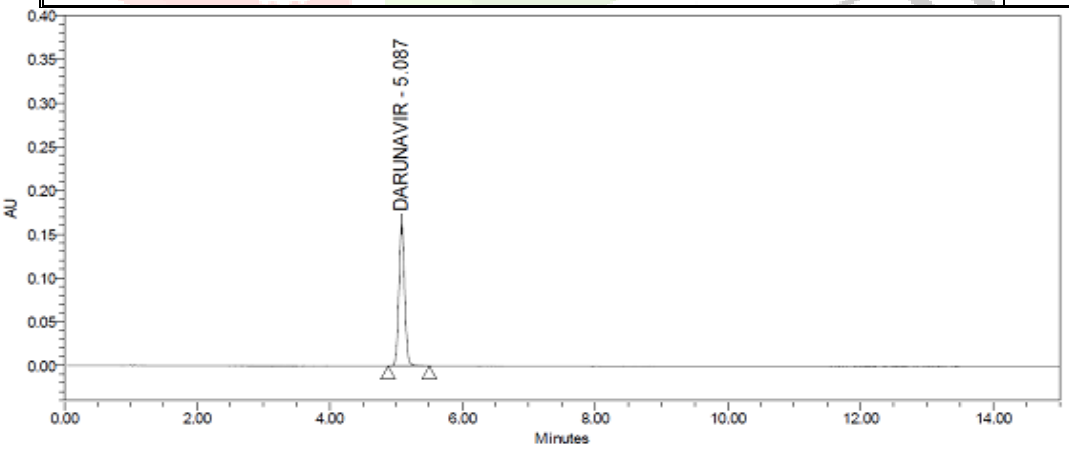

Fig. 5.5 (c): Chromatogram of Darunavir sample solution

The result of system precision, method precision and intermediate precision expressed as % RSD were found to be 0.33, 0.7 and 0.6 for Darunavir in **Table 5.5 (a,b & c)** respectively**.** According to acceptance criteria the % RSD for the standard and its impurity should not be more than 2. Hence the method was found to be précised.

5.6 Limit of Detection and Limit of Quantitation:

Table 5.6: LOD and LOQ for Darunavir

Sr. No	Standard Deviation	slope	Parameters	Result
1	1.388861	12805	LOD	0.000358
2			LOQ	0.001085

Based on the standard deviation of response and slope of Darunavir, LOD and LOQ were determined. The observation of LOD and LOQ studies are shown in the Table 5.6**.** Darunavir's LOD was determined to be 0.000358 and its LOQ to be 0.001085 g/mL, respectively.

5.7 Solution stability:

Table 5.7: Analytical Standard and Sample Solution Stability at 15°C and 25°C

According to the suggested approach, a standard and sample solution was made, then it was examined initially and then again at various time intervals while being kept at the ambient temperature. The observations are shown in Table 5.7. The % difference for the area count of Darunavir was found to be less than 2.0; hence the standard and sample solutions were stable for 24 hrs at room temperature.

Time	Standard and Sample solution (15°C)				Standard and Sample solution (25°C)			
	Standard Solution Stability		Sample Solution Stability		Standard Solution Stability		Sample Solution Stability	
	Response (area) (µV)	Cumulative % RSD	% Assay	Absolute % Difference	Response (area) (µV)	Cumulative % RSD	% Assay	Absolute % Difference
Initial	987789.2	NA	98.6	NA	982334	NA	98.8	NA
12 HRS	996991	1.2	99.2	-0.89	983466	1.0	99.2	-0.89
20 HRS	978761	1.6	98.2	0.41	988291	1.1	99.1	-0.51
24 HRS	985680	1.3	100.0	-1.68	999622	1.0	97.4	0.90

5.8 Robustness:

Table 5.8: Robustness for Darunavir

Changes in parameters	Values	Retention Time	Theoretical Plates	Symmetry Factor	%RSD	% Assay	% Absolute Difference
*Control	As per method	5.210	15254	1.0	0.3	100.0	NA
Wavelength (265nm)	260nm	5.209	15215	1.0	0.3	102.1	0.0
	270nm	5.210	15270	1.0	0.4	102.0	0.1
Flow Rate (1.5mL/Min)	1.6mL/min	5.004	10023	1.0	0.4	99.7	0.4
	1.4mL/min	5.682	12124	1.0	0.5	99.5	1.0
Column temp. (60°C)	55°C	5.298	13392	1.0	0.6	101.6	0.5
	65°C	5.041	10287	1.0	0.7	98.4	0.1
pH (4.0)	3.8 pH	5.011	9227	0.9	0.8	100.0	0.1
	4.2 pH	5.288	9251	0.9	0.6	98.8	1.3

In these study deliberate variations in method parameters were done i.e. Modification in wavelength (± 5nm), Modification in flow rate (± 0.1 ml/min), Modification in column temperature (± 5°C), and Modification in buffer pH (+0.2unit).

From the results of robustness study in Table 5.8, it can be concluded that the absolute difference of % assay in each modified condition for % assay of initial and after change should be ± 2.0%. Hence proposed method was found to be robust.

6. CONCLUSIONS:

The results of the investigations mentioned above allow us to draw the conclusion that the suggested HPLC approach may be utilised to estimate the assay in Darunavir.

Darunavir determination using the HPLC method provided are reliable, accurate, and precise results. Darunavir in tablet form can be regularly subjected to quality control examination using this technique. The amount discovered using the suggested procedures was in line with the label claim for the formulation. The proposed methodologies can be used for routine estimate of tablet dosage forms because the calculated standard deviation and coefficient of variation were both adequately low.

7. ACKNOWLEDGEMENT:

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