ORIGINAL ARTICLE

A New Validated RP-HPLC Method for the Estimation of Darunavir Ethanolate in Bulk and Tablets

A. N. Kavitha[1], Raman Dang[2], K. Janakiraman[3]

[1]Department of Pharmaceutics, Krupanidhi College of Pharmacy, Bengaluru, Karnataka, India, [2]Principal, KLE College of Pharmacy, Bengaluru, Karnataka, India, [3]Department of Pharmacy, Faculty of Engineering and Technology, Annamalai University, Chidambaram, Tamil Nadu, India

Abstract

Objective: Darunavir ethanolate is the last United States Food and Drug Administration approved protease inhibitor (PI). The drug is used along with other PIs (ritonavir/cobicistat) for the effective management of human immunodeficiency virus (HIV) HIV-1 infection. Darunavir ethanolate exerts its action by binding non-competitively to HIV protease enzyme. The main objective of the present research work was to develop a new profound and novel reverse-phase high-performance liquid chromatography (RP-HPLC) for the estimation of darunavir ethanolate. **Methods:** As per the guidelines of the Food and Drug Administration and International Council for harmonization, the method was validated. The HPLC analysis was performed on the waters 2695 equipped with symmetry C_{18} column 3.5 µm, 150 mm × 4.6 mm, with a mixture of acetonitrile:0.1% phosphate buffer (50:50 V/V) as the mobile phase, at the flow rate of 1 ml/min. The total run time was 5 min and the detection was performed at the wavelength (λ) of 262 nm. **Results:** The retention time for darunavir ethanolate was found to be 2.269 min. The standard curves were obtained with R^2 0.9997 and linear at the concentration range of 8–120 µg/ml. The limit of quantitation and limit of detection for darunavir ethanolate were found to be 0.08 µg/ml and 0.8 µg/ml, respectively. The method's accuracy was tested by percentage recovery tests and found to be 100.3%. The results obtained were within the accepted standards for linearity, accuracy, precision, specificity, and robustness. **Conclusion:** The proposed RP-HPLC method can be applied for the routine analytical estimation of darunavir ethanolate in bulk and tablet formulations.

Key words: Acquired immunodeficiency syndrome, darunavir ethanolate, human immunodeficiency virus, international council for harmonization guidelines, reverse-phase high-performance liquid chromatography, validation

INTRODUCTION

Human immunodeficiency virus (HIV) is a type of retrovirus, invades T-immune cells (CD4 cells) in the human body. The untreated HIV infection may decrease CD4 cell count and leads to an advanced disease stage called acquired immunodeficiency syndrome (AIDS) and various other life-threatening infections.[1] HIV/AIDS treatment is called antiretroviral therapy (ART), where ART involves taking a combination of HIV drugs.[2] The HIV medicines help in preventing the multiplication of virus in the host cell further reduces viral load in the body to an undetectable level.[3,4] The reduced viral load improves the immune system by raising the CD4 cell count. Combinational therapy with three or four antiretroviral drugs is known as highly active antiretroviral therapy (HAART).[5] HAART is recognized as the most effective treatment method for AIDS.

There are six classes of antiretroviral agents:

1. Nucleoside reverse transcriptase inhibitors (NRTIs)
2. Non-nucleoside reverse transcriptase inhibitors (NNRTIs)
3. Protease inhibitors (PIs)
4. Integrase inhibitors (INSTIs)

Address for correspondence:
A. N. Kavitha, Department of Pharmaceutics, Krupanidhi College of Pharmacy, Bengaluru - 560 035, Karnataka, India. Mobile: +91-9986656381.
E-mail: kavithareddykcp@gmail.com

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5. Fusion inhibitors (FIs)
6. Chemokine receptor antagonists (CCR5s)

NRTIs

NRTIs are the primary class existing for the treatment of HIV infection. They exhibit activity against both HIV-1 and HIV-2. These agents interrupt the HIV replication process by hindering HIV reverse transcriptase and terminating the deoxyribonucleic acid (DNA). NRTIs are structurally identical to the DNA nucleotide bases that integrate into the structure of the viral DNA.[6] The drugs include in this class are emtricitabine and tenofovir, etc., which acts against the hepatitis-B virus.

NNRTIs

NNRTIs are also preferred in initial treatment regimens. They exhibit potent activity against HIV-1 and this agent is also interrupt the HIV reverse transcriptase by binding to the allosteric site of HIV and induces conformational changes by binding to hydrophobic site, p66 subunit of the heterodimer of HIV, and this non-competitive inhibition leads to the conformational enzyme transition that alters the dynamic site and restricts virus activity. The drugs under this class are nevirapine, rilpivirine, etc.

PIs

PIs play a vital role in the HAART. The PIs are competitive inhibitors that bind to the HIV protease and block the proteolytic cleavage of protein precursor. The drugs included in this class are atazanavir, nelfinavir, indinavir, etc.

INSTIs

HIV integrase has a significant role in HAART. HIV integrase catalyzes the major biochemical step that incorporates the viral DNA into host chromosomal DNA by binding to metallic ions at the dynamic site and halts the strand transfer reaction. The drugs included in this class are dolutegravir, elvitgravir, etc.

FIs

FIs bind to gp41 subunit of viral envelop glycoprotein and prevent the entry of HIV to the CD4 cells so, it is also called as entry inhibitors. Thus, interferes with the fusion of viral and cellular membrane. The only drug included in this class is enfuvirtide.

CRAs

CRAs block the V3 loop interaction by binding reversibly to the CCR5 co-receptor. Thus inhibits the fusion of the cellular membranes. Maraviroc is the only drug discovered in this class.

Darunavir belongs to second-generation PI, where it is effective against HIV1.[7] Darunavir shows its action by binding non-competitively to HIV protease enzyme. The protease enzyme is responsible for the breaking of HIV encoded Gag-Pol protein in a host cell infected with a virus and leads to the formation of the mature infectious viral particles.[8-10] For the effective management of HIV-1 infection, darunavir is administered with other PIs. The co-administration of cytochrome P450 enzyme inhibitor (ritonavir) with darunavir, there is a significant decrease in the viral load and enhanced CD4 cell count. Darunavir ethanolate is [(3aS, 4R, 6aR)-2, 3, 3a, 4,5,6a-hexahydrofuro[2,3-b] furan-4-yl] *N*-[(2*S*,3*R*)-4-[(4-aminophenyl)sulfonyl-(2-methylpropyl)amino]-3-hydroxy-1-phenylbutan-2yl] carbamate; ethanol and the structure is shown in Figure 1. The molecular formula of darunavir ethanolate is $C_{29}H_{43}N_3O_8S$, with a molecular weight of 593.7 g/mol.

Analytical method development is a crucial step in product development and the validation of the developed methods as per the guidelines ensures the suitability of the method for intended purpose. The literature survey revealed that few methods have been reported for the estimation of darunavir ethanolate in bulk and dosage forms.[11] The aim of the present study was to develop and validate a new simple, sensitive, and rapid reverse-phase high-performance liquid chromatography (RP-HPLC) method for the determination of darunavir ethanolate in bulk and pharmaceutical dosage form.

Method validation has established a significant consideration in the literature, regulatory agencies, and industrial committees, the components of typical analytical performance characteristics that are analyzed during method validation are specificity, accuracy, precision, linearity, robustness, and sensitivity as per the ICHQ2 (R1) guidelines.[12]

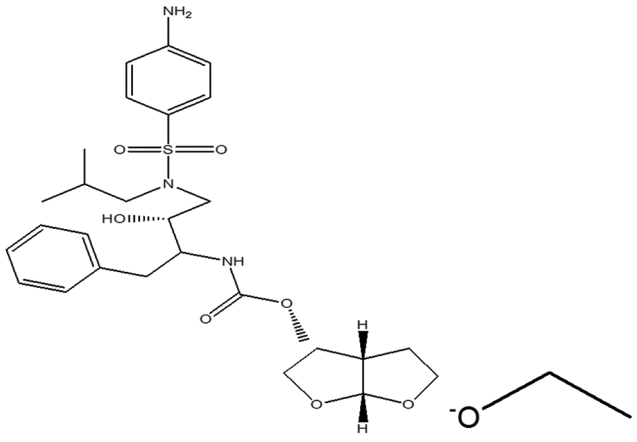

Figure 1: Darunavir ethanolate chemical structure

MATERIALS AND METHOD DEVELOPMENT

Instrumentation

A Waters 2695 HPLC system was used for the liquid chromatography method development and validation, the equipment is equipped with a symmetry C_{18} column, 3.5 µm, 150 mm × 4.6 mm equipped with an autosampler and the pump with ultraviolet/visible spectroscopy detector (operated at 262 nm). In addition, microliter syringe and micropore filtration assembly were used in the study. The data processing and evaluation were done with empower 2 software.

Chemicals and reagents

Darunavir ethanolate was a kind gift from Aurobindo Pharma Ltd., Hyderabad, India. All reagents were of HPLC grade. Acetonitrile (ACN) and orthophosphoric acid were supplied by Sigma-Aldrich, Germany.

Preparation of buffer and mobile phase

Preparation of buffer (0.1% orthophosphoric acid [OPA])

In a cleaned and dried 1000 ml volumetric flask, 1 ml of HPLC grade OPA was measured using micropipette and added to 900 ml of HPLC grade Milli-Q water using calibrated measuring jars, mixed well and made up the volume with the same and added few drops of HPLC grade tri ethyl amine to adjust the pH to 4.0 ± 0.05 and filtered through 0.45 µ filter under vacuum and sonicated for about 10 min to degas the buffer.

Preparation of mobile phase

The mobile phase was prepared by mixing 50:50 v/v ACN and 0.1% phosphate buffer and filtered through a 0.45 µ membrane filter. The prepared mobile phase was degassed by sonication for about 10 min.

Preparation of diluent

The diluents were prepared by mixing water and ACN in the ratio of 50:50 v/v, filtered through 0.45 µ filters under vacuum.

Preparation of standard stock solution

The standard stock solution was prepared by transferring accurately weighed 80 mg of darunavir ethanolate into a clean dry volumetric flask of 100 ml capacity. The drug was dissolved by adding 65 ml of diluent and then sonicated for 15 min and diluent filled up to the mark with diluent to get the final concentration of 800 µg/ml.

Preparation of standard solution

The working standard solution was prepared by transferring 5 ml of the standard stock solution into a 50 ml volumetric flask. The volume was made up to the mark with the diluent (80 µg/ml).

Method development

Chromatographic conditions were optimized by modifying the chromatographic variables such as use of different columns, mobile phase composition, and flow rates. Experiments were carried out at constant conditions such as appropriate wavelength 262 nm, injection volume of 10 µl, and run time of about 5 min throughout the trials to achieve the best possible separation and resolution. The conditions which produce the best resolution, United States Pharmacopeia (USP) plate count and tailing factor, were selected for the estimation. The resultant chromatograms were verified, and chromatographic parameters such as resolution, USP plate count, and tailing factor were calculated [Tables 1 and 2].

METHOD VALIDATION

System suitability tests (SST)

The system stability study was conducted by consecutively injecting five consecutively replicate injections of 10 µL (80 µg/ml) of the standard solution of darunavir into the chromatographic system using optimized chromatographic conditions to assess the system suitability parameters. The parameters used in the SST report are as follows: (1) Number of theoretical plates or efficiency (N), (2) capacity factor (K), (3) separation or relative retention (α), (4) resolution (Rs), (5) tailing factor (T), and (6) relative standard deviation (RSD).

Construction and linearity of calibration curve

The different concentrations of working standard solutions were prepared by diluting the standard solution darunavir with the mobile phase to get the final concentration between 8 and 120 µg/ml. Ten microliters of each dilution at 1 ml/min flow rate was injected into the column. The darunavir content in the eluent was measured at 262 nm and the respective chromatograms were obtained. Each dilution was injected 6 times into the column and mean areas were determined. A graph was obtained by plotting standard concentration in Abscissa and mean peak area in ordinate. Further, the linear relationship between peak area and concentration was assessed by statistical determination of a regression line using the least square methodology.

Table 1: Selection of chromatographic conditions

Trial. no	Composition of mobile phase	Buffer	Column (dimensions and pore size)	Flow rate	Injection volume	Diluent	Observation
1	ACN:Buffer (80:20)	1 ml TEA is dissolved water adjusted pH to 7.0 with orthophosphoric acid	Luna C18 250×4.6 mm, 5 µ	1.00 ml/min	10 µl	Water:ACN (50:50 v/v)	Peak tailing is not within the limit
2	ACN:Buffer (70:30)	1 ml TEA is dissolved water adjusted pH to 7.0 with orthophosphoric acid	Luna C_{18} 250×4.6 mm, 5 µ	1.00 ml/min	10 µl	Water:ACN (50:50 v/v)	Peak plate count and tailing not within the limit
3	ACN:Buffer (60:40)	1 ml TEA is dissolved water adjusted pH to 7.0 with orthophosphoric acid	Symmetry C_{18} 150×4.6 mm, 3.5 µ	1.00 ml/min	10 µl	Water:ACN (50:50 v/v)	Peak tailing is not within the limit
4	ACN:Buffer (70:30)	0.1% orthophosphoric acid	Symmetry C_{18} 150×4.6 mm, 3.5 µ	1.00 ml/min	10 µl	Water:ACN (50:50 v/v)	Blank peaks were observed
5	ACN:Buffer (60:40)	0.1% orthophosphoric acid	Symmetry C_{18} 150×4.6 mm, 3.5 µ	1.00 ml/min	10 µl	Water:ACN (50:50 v/v)	Peak splitting is observed
6	ACN:Buffer (50:50)	0.1% orthophosphoric acid	Symmetry C_{18} 150×4.6 mm, 3.5 µ	1.00 ml/min	10 µl	Water:ACN (50:50 v/v)	All suitability conditions are within the limit

ACN: Acetonitrile

Table 2: Optimized chromatographic conditions for proposed high-performance liquid chromatography method

Instrument	Waters 2695, high-performance liquid chromatography
Mobile phase	0.1% phosphate buffer:acetonitrile 50:50%V/V
Flow rate	1 ml/min
Column	Symmetry C_{18} column 150 mm×4.6 mm, 3.5 µm
Detector wave length	262 nm
Column temperature	30°C
Injection volume	10 µl
Run time	5 min
Diluent	Water:acetonitrile (50:50)
Mode of separation	Isocratic mode

Precision

System precision (repeatability/intra-day variation)

The system precision study was demonstrated by injecting 10 µl solution of standard preparations 6 times into the chromatographic system and chromatograms were documented. Darunavir peak areas were calculated and results were expressed as % RSD.

Method precision

The method precision of the test study was demonstrated by injecting 10 µl solution of sample preparations 6 times into the chromatographic system and chromatograms were documented. Darunavir peak areas were calculated and results were expressed as % RSD.

Intermediary/inter-day variation (ruggedness)

The intermediate precision of the process was demonstrated by injecting 10 µl solution of standard preparations 6 times into the chromatographic system on different days and chromatograms were documented. Darunavir peak areas were calculated and results were expressed as % RSD.

Accuracy (recovery)

The accuracy of the technique was studied by % recuperation over its range by making three distinct concentrations at 50%, 100%, and 150% levels using the standard addition method, where test preparations were spiked with a known measure of standard and later, each concentration was injected triplicate into the chromatographic system and chromatograms were documented. The darunavir % recoveries obtained from each level were calculated.

Robustness

The robustness of the proposed system was assessed by deliberately varying the chromatographic settings such as

wavelength, compositions of the mobile phase, column temperature, and flow rate; as per the test method, the standard solutions were prepared and injected triplicate into the chromatographic system at variable conditions such as flow rate at ± 0.1 ml/min, mobile organic phase composition by ±10%, wavelength by ±5 nm, and column temperature by ± 5°C. System suitability components were assessed from the obtained chromatograms.

Specificity (interference studies)

The specificity of the technique for the interference of the placebo and the blank was conducted by injecting, placebo, standard, sample solutions, and blank in triplicate as per the test method. The proposed RP-HPLC method specificity, also assessed by comparing the recorded chromatograms from standard, sample solutions, and blank.

Sensitivity – limit of quantification (LOQ) and limit of detection (LOD)

The lowest concentration that can be quantified with a specified level of accuracy and precision is called as LOQ. The measurable response obtained from the lowest concentration of the analyte is called as LOD. The LOQ and LOD of darunavir ethanolate were determined by analyzing the different solutions of drug and measuring signal-to-noise ratio.

Analysis of a marketed formulation

To estimate the darunavir ethanolate content in the marketed tablet (Daruvir 600), 20 tablets were weighed and the mean weight of the tablet was calculated. The tablets were finely powdered and tablet triturate equivalent to 80 mg was weighed and transferred to 100 ml volumetric flask containing 60 ml of diluent and the contents were sonicated for 30 min. The volume made up to 100 ml with the diluent. The solution was centrifuged at 3000 rpm for 5 min to obtain the clear supernatant liquid. The resultant stock solution was diluted suitably to get the final concentration of 80 µg/ml. The solution was estimated by the above developed HPLC method. The drug content was estimated using the regression equation.[13]

RESULTS AND DISCUSSION

Linearity

The linearity indicates the correlation between the known concentration of the analyte and the response. The mean peak area [Table 3] obtained from the HPLC was plotted against corresponding concentrations to obtain the calibration graph obtained by plotting relative concentration versus mean peak area. The linear graph obtained was presented in Figure 2. The different chromatograms were obtained for the various concentrations of the analyte [Figures 3-8]. The linearity study results gave a linear relationship over the concentration range of 8–120 µg/ml for darunavir ethanolate. The linear equation y = 47783x + 21441 was obtained and goodness-of-fit (R^2) value was found to be 0.9997. The regression analysis indicates the linearity between the concentrations of the analyte with the area under the peak [Table 4].

Precision (repeatability/intra-day precision)

The % RSD of the peak areas for darunavir ethanolate is depicted in Tables 5 and 6.

Accuracy

The accuracy of the proposed method was estimated by the standard recovery studies. The results obtained for the accuracy study were tabulated [Table 7]. The percentage recovery of all the three levels was in the range of 99.9–100.8% and the % RSD was found to be 0.01–0.13%. The satisfactory results gained for the accuracy study show that the proposed HPLC method is accurate.

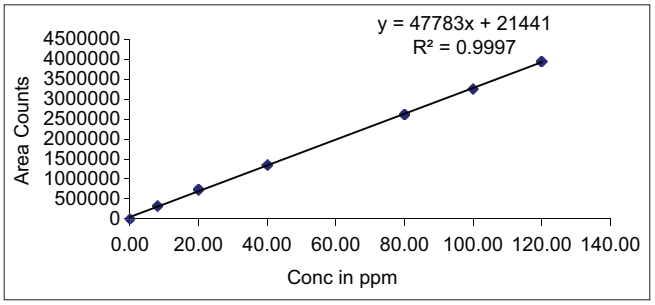

Figure 2: Standard calibration graph

Table 3: Data for the linearity studies

Concentration µg/ml	Peak area
8.00	326971
20.00	731459
40.00	1347935
80.00	2619390
100.00	3263458
120.00	3946340

Table 4: Regression characteristics of the developed method

Parameter	Results
Linearity range (µg/ml)	8–120
Regression equation (*y*=mx+b)	*y*=47783x+21441
Slope(m)	47783
Intercept(b)	21441
Correlation coefficient (r^2)	0.9997

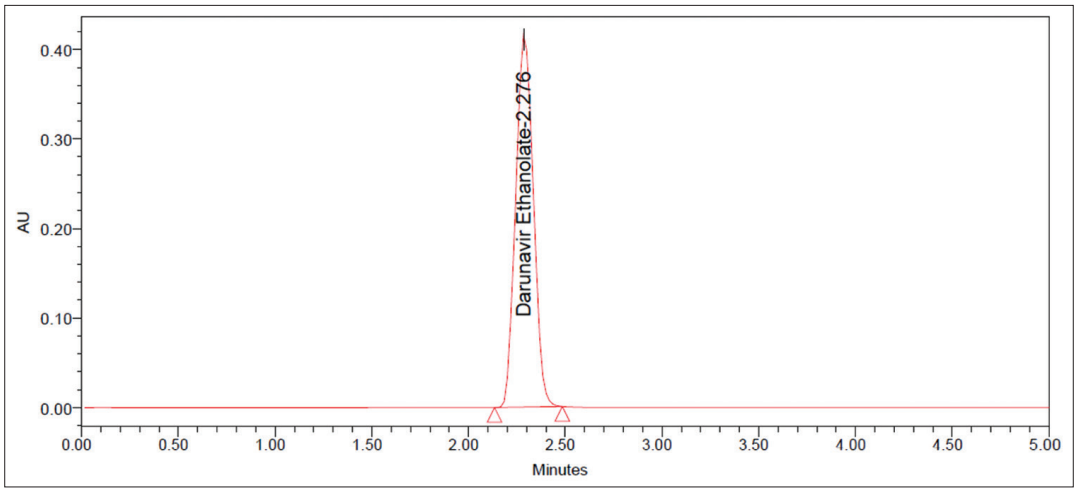

Figure 3: Darunavir ethanolate high-performance liquid chromatography chromatogram of 25% level of linearity solution

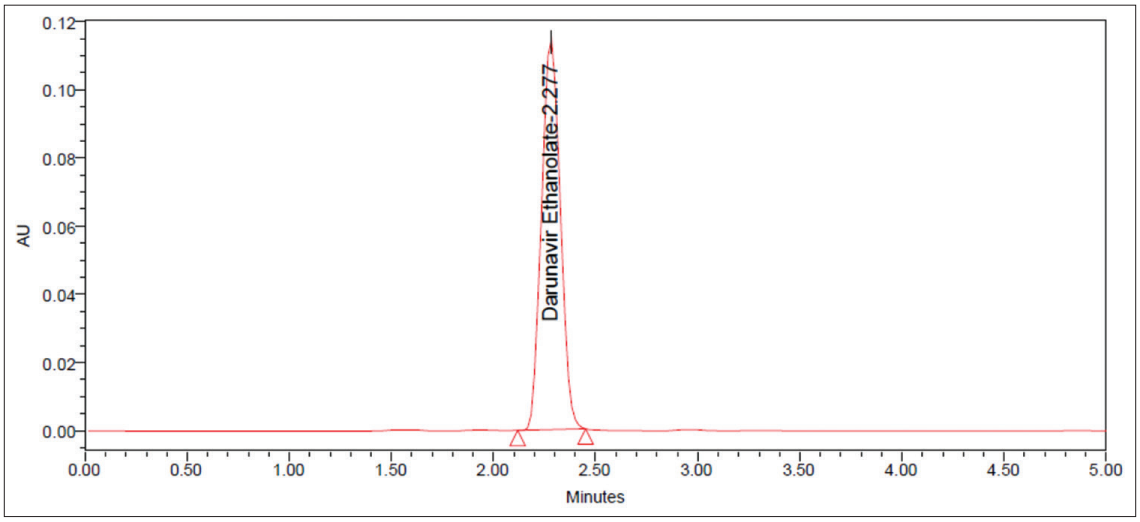

Figure 4: Darunavir ethanolate high-performance liquid chromatography chromatogram of 50% level of linearity solution

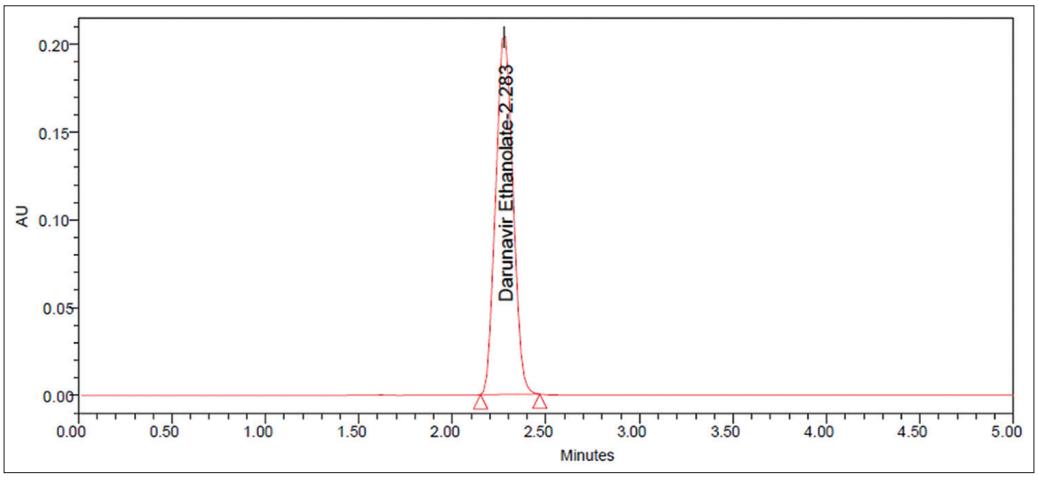

Figure 5: Darunavir ethanolate high-performance liquid chromatography chromatogram of 75% level of linearity solution

Robustness

Robustness evaluation examines the outcome of intentional deviations in the method parameters associated with the analytical procedure. It was found that the system suitability parameters were within limits at all variable conditions. From the results obtained [Table 8], it can be concluded; the developed RP-HPLC technique is robust toward small deviations.

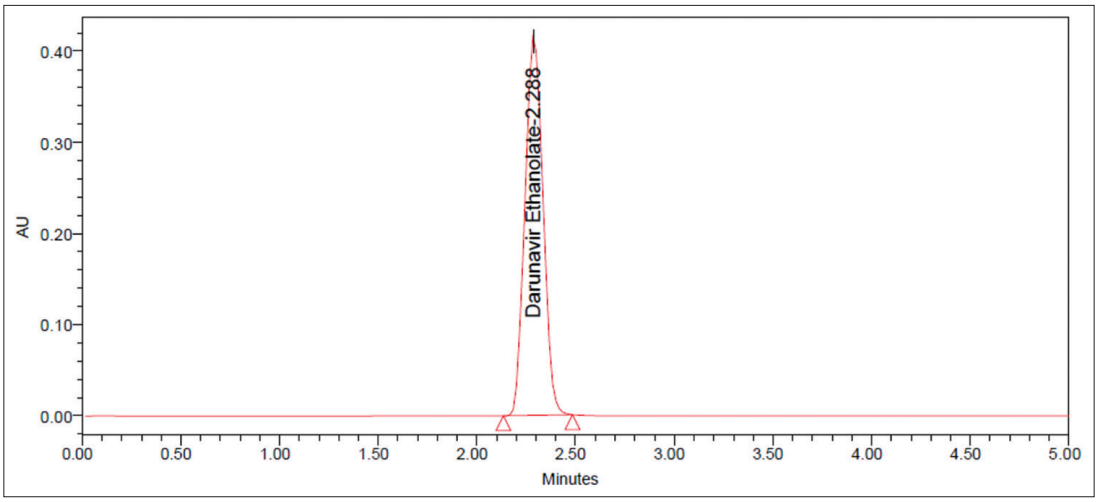

Figure 6: Darunavir ethanolate high-performance liquid chromatography chromatogram of 100% level of linearity solution

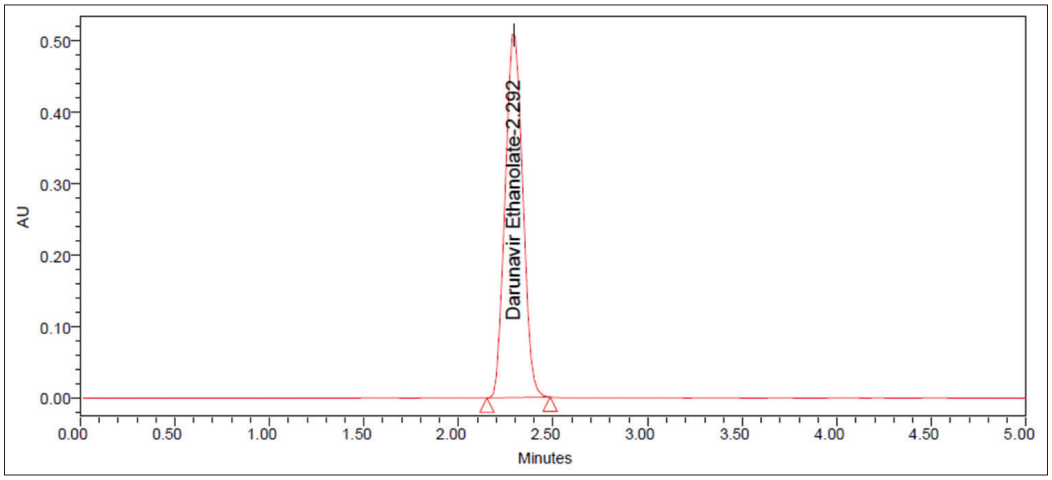

Figure 7: Darunavir ethanolate high-performance liquid chromatography chromatogram of 125% level of linearity solution

Table 5: Results of the repeatability studies (intraday precision)

Number of injection	Retention time (min)	Peak area
1	2.271	2619390
2	2.278	2618967
3	2.262	2617472
4	2.267	2619298
5	2.274	2619356
6	2.276	2621517
Statistical parameters	Mean	2619333
	SD	1293.99
	% RSD	0.05

Table 6: Results of the precision studies

Number of injection	Retention time (min)	Peak area
1	2.269	2622418
2	2.273	2621582
3	2.277	2614570
4	2.279	2620269
5	2.281	2619321
6	2.270	2621651
Statistical parameters	Mean	2619968
	SD	2866.62
	% RSD	0.11

Specificity (interference studies)

The chromatograms obtained from the sample and standard solutions and blank were obtained and outcomes of specificity studies are reported in Table 9.

LOQ and LOD

LOQ value was found to be 0.8 µg/ml and the LOD value was found to be 0.08 µg/ml. The low LOQ and LOD values obtained indicate that the developed RP-HPLC method is sensitive.

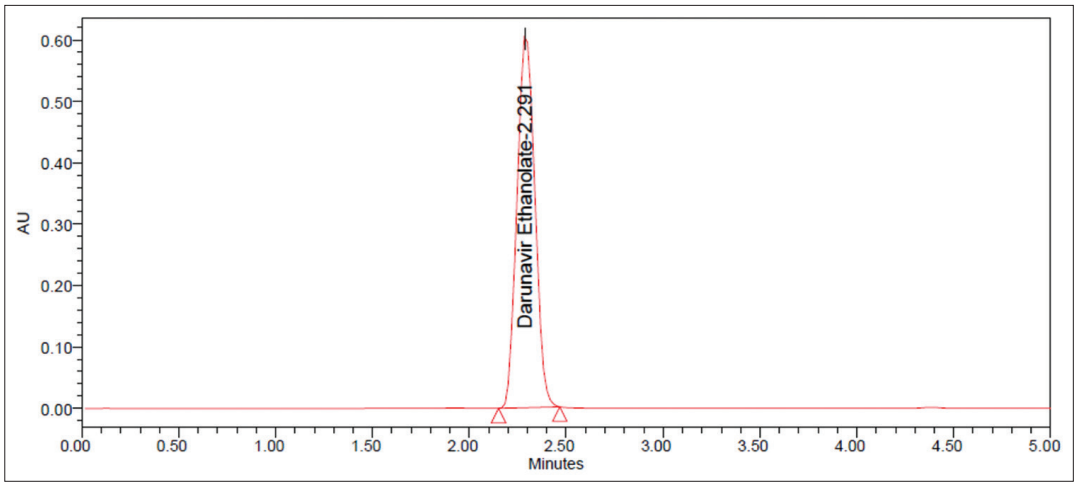

Figure 8: Darunavir ethanolate high-performance liquid chromatography chromatogram of 150% level of linearity solution

Table 7: Data of accuracy studies

S. No	% Level of test concentration.	Peak area	Amount added (µg/ml)	Amount recovered (µg/ml)	% recovery	Mean % recovery	% RSD*
1	50	1347791	40.8	40.80	100.9	100.8	0.13
2	50	1347841	40.9	40.90	100.7		
3	50	1347861	40.8	40.80	100.9		
4	100	2619474	80.00	80.00	100.0	99.9	0.09
5	100	2619478	80.05	80.05	99.9		
6	100	2619564	80.15	80.15	99.8		
7	150	3946370	120	120.00	100.4	100.4	0.01
8	150	3946370	120	120.00	100.4		
9	150	3946457	120	120.00	100.4		

Table 8: Data of robustness studies

Parameter	Optimized condition	Used condition	Peak area	Retention time	Plate count	Tailing factor
Flow rate (±0.1 ml/min)	1 ml/min	0.9 ml/min	1646457	3.172	2681	1.06
		1 ml/min	1684721	3.180	2612	1.06
		1.1 ml/min	1684587	3.188	2602	1.06
Column temp. (±5°C)	30°C	25°C	2218247	1.998	2057	1.06
		30°C	2218461	1.999	2109	1.05
		35°C	2218725	2.000	2111	1.06
Mobile phase composition (5%v/v)	50:50	40:60	2218287	1.997	2057	1.06
		45:55	2218247	2.003	2041	1.06
		55:45	2218254	2.005	2141	1.06

Table 9: Results of specificity studies

S. No	Solution	RT (min)	Peak area
1	Blank	-	-
2	Standard	2.22	2619360
3	Sample	2.269	2622418

Analysis of a marketed formulation

The marketed darunavir tablets were analyzed using the proposed HPLC method and the obtained results are presented in Table 10. The chromatogram obtained for darunavir ethanolate from the marketed product is shown

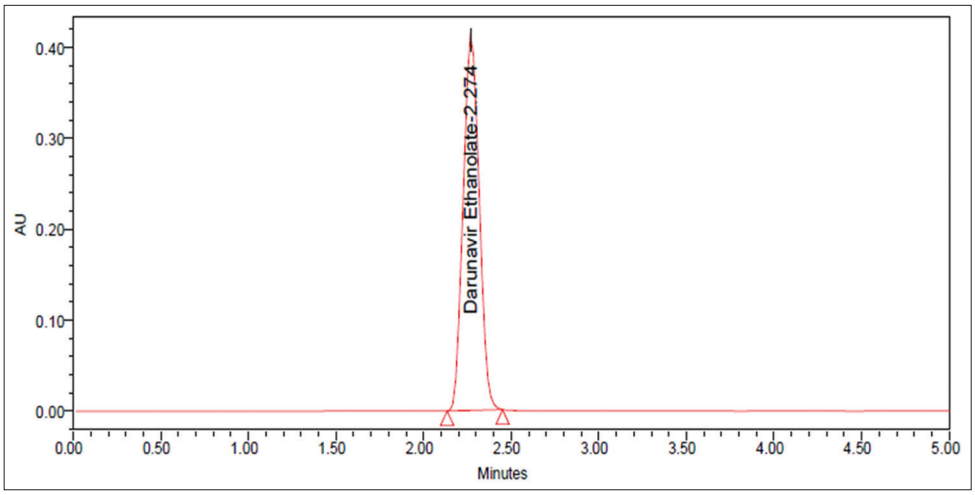

Figure 9: Darunavir ethanolate high-performance liquid chromatography chromatogram of marketed product

Table 10: Results of marketed formulation

Brand name	Daruvir 600
Label claim (mg)	600 mg
Test concentration (µg/ml)	80.0
Mean amount estimated (µg/ml) (*n*=6)	80.02
% Assay	99.97
% RSD	0.44

in Figure 2. The drug content was found to be 99.97% and the studies showed that there is no interference from the excipients present in the formulation [Figure 9].

CONCLUSION

The results of this investigation reveal that, by applying the proposed RP-HPLC technique, the retention times of darunavir was found to be 2.269 min. Quantitative linearity was obeyed in the concentration range of 8–120 µg/ml with a correlation coefficient value of 0.999. The % RSD values obtained from the precision studies were also found to be <2, which specify the precise method. The high % recoveries specify that the developed method was highly accurate. The low values of LOQ and LOD indicate the high sensitivity of the proposed method. The absence of interfering peaks observed in the chromatogram of blank and placebo interference studies indicates the specificity of the proposed RP-HPLC method. From this study, it is concluded that the developed RP-HPLC technique was found to be rapid, precise, simple, accurate, and valuable for the tedious analysis of darunavir ethanolate in pharmaceutical dosage forms and bulk. As per ICH guidelines, the achieved reports were satisfactory.

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