Rp- Hplc Method Development and Validation for Estimation of Darolutamide with Bulk Form and Marketed Pharmaceutical Dosage Forms

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ABSTRACT :
A simple, rapid, specific and accurate reverse phase high performance liquid chromatographic method has been developed for the validated of Darolutamide in bulk as well as in marketed pharmaceutical dosage form. This separation was performed on a Symmetry ODS C18 (4.6mm×250mm, 5µm) column with Methanol: Phosphate Buffer (35:65%) v/v as mobile phase at a flow rate of 1.0 mL min−1 with UV detection at 235 nm; the constant column temperature was Ambient. The run time under these chromatographic conditions was less than 8 min. The retention time of Darolutamide was found to be 2.293min. The calibration plot was linear over the concentration range of 6–14 µg mL−1 with limits of detection and quantification values of 1.2 and 3.6 ng mL−1 respectively. The mean % assay of marketed formulation was found to be 99.86%, and % recovery was observed in the range of 98-102%. Relative standard deviation for the precision study was found <2%.The developed method is simple, precise, specific, accurate and rapid, making it suitable for estimation of Darolutamide in bulk and marketed pharmaceutical dosage form dosage form.
Keywords: Darolutamide, RP-HPLC, Method Development, Validation, ICH Guidelines.

I.INTRODUCTION

Darolutamide is a third generation, oral nonsteroidal antiandrogen used to treat nonmetastatic castration-resistant prostate cancer. Darolutamide is associated with a low rate of serum enzyme elevation during therapy, but has not been linked to cases of clinically apparent liver injury with jaundice[1]. Darolutamide is indicated for the treatment of adults with non-metastatic castration-resistant prostate cancer (nmCRPC) and metastatic hormone-sensitive prostate cancer (mHSPC) in combination with docetaxel.The actions of androgens on androgen receptors (AR) potentiate the growth and survival of prostate cancer cells. Darolutamide competitively inhibits androgens from binding to their receptors, inhibiting AR nuclear translocation, as well as AR-mediated transcription. The end result of these processes is a decrease in prostate cancer cell proliferation and tumour size. Its main metabolite, keto-Darolutamide, shows similar pharmacological activity to the parent drug, Darolutamide[2]. Darolutamide has been found to bind more tightly to the AR receptor than Apalutamide and enzalutamide, which are other androgen receptor antagonists. Darolutamide can act as a progesterone receptor (PR) antagonist in the laboratory setting with approximately 1% activity when compared to its actions at the androgen receptor. The clinical relevance is not known at this time[3].The IUPAC Name of Darolutamide is 5-O-[1-[3, 3-diphenylpropyl (methyl) amino]-2-methyl propan-2-yl] 3-O-methyl 2, 6-dimethyl-4-(3-nitro phenyl)-1, 4-dihydro pyridine-3, 5-dicarboxylate. The Chemical Structure of Darolutamide is shown in follows

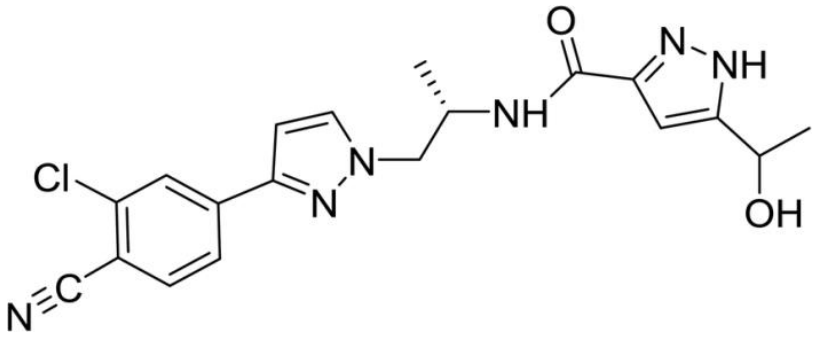

Fig-1: Chemical Structure of Darolutamide

The considerable literature surveydisclose that the analytical methods reported for estimation of Darolutamidealone and in combination with other drug, but no analytical methods was reported for estimation of Darolutamide. So, it was thought of interest to develop simple, accurate, precise and reproducible RP-HPLC method for estimation of Darolutamidein their bulk form and Marketed Pharmaceutical Dosage forms[28-30]. Methods were validated as per ICH guidelines[27] [Q2 (R1)].

II. EXPERIMENTAL WORK

Table-1: Instruments Used

S.No.	Instruments and Glass wares	Model
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detectors.
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Labman

Table-2: Chemicals Used

S.No.	Chemical	Brand Names
1	Darolutamide	Local Market
2	Water and Methanol for HPLC	LICHROSOLV (MERCK)
3	Acetonitrile for HPLC	Merck

HPLC Method Development:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Darolutamide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.1ml of the above Darolutamide stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines[27].

Mobile Phase Optimization:

Initially the mobile phase tried was Methanol and Methanol: Water with varying proportions. Finally, the mobile phase was optimized to Methanol: Phosphate Buffer in proportion 35:65% v/v.

Optimization of Column:

The method was performed with various C18 columns like, X- bridge column, Xterra, and C18 column. Symmetry ODS C18 (4.6mm x 250mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1.0ml/min flow.

Preparation of Potassium dihydrogen Phosphate (KH2PO4) buffer (pH-3.6):
Dissolve 6.8043 of potassium dihydrogen phosphate in 1000 ml HPLC water and adjust the pH 3.6 with diluted orthophosphoric acid. Filter and sonicate the solution by vacuum filtration and ultra-sonication[4].

Preparation of Mobile Phase:
Accurately measured 350 ml (35%) of Methanol, 650 ml of Phosphate buffer (65%) were mixed and degassed in digital ultra sonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:
The Mobile phase was used as the diluent[5].

Method Validation Parameters
System Suitability
Accurately weigh and transfer 10 mg of Darolutamide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.1ml of the above Darolutamide stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:
The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Specificity:
Preparation of Standard Solution:
Accurately weigh and transfer 10 mg of Darolutamide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.1ml of the above Darolutamide stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of Sample Solution:
Weight 10 mg equivalent weight ofDarolutamide sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
Further pipette 0.1ml of Darolutamide above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:
Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

Linearity and Range:
Accurately weigh and transfer 10 mg of Darolutamide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (6ppm of Darolutamide):
Take 0.6ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – II (8ppm of Darolutamide):
Take 0.8ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – III (10ppm of Darolutamide):
Take 0.1ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – IV (12ppm of Darolutamide):
Take 0.12ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – V (14ppm of Darolutamide):
Take 0.14ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Procedure:
Inject each level into the chromatographic system and measure the peak area.
Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient[6].

Precision
Repeatability
Preparation of Darolutamide Product Solution for Precision:
Accurately weigh and transfer 10 mg of Darolutamide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.1ml of the above Darolutamide stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:
The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Intermediate Precision:
To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:
Analyst 1:
The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Analyst 2:
The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy:
For preparation of 50% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Darolutamide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.05ml of the above Darolutamide stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

For preparation of 100% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Darolutamide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.1ml of the above Darolutamide stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

For preparation of 150% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Darolutamide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.15ml of the above Darolutamide stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:
Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Darolutamide and calculate the individual recovery and mean recovery values.

Limit of Detection and Limit of Quantification (LOD & LOQ):
Preparation of 0.95µg/ml Solution (For LOD):
Accurately weigh and transfer 10 mg of Darolutamide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.0095ml of the above Darolutamide stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of 2.9µg/ml Solution (For LOQ):
Accurately weigh and transfer 10 mg of Darolutamide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.029ml of the above Darolutamide stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Robustness:
The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results. .

For preparation of Standard solution:
Accurately weigh and transfer 10 mg of Darolutamide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.1ml of the above Darolutamide stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Effect of Variation of Flow Conditions:
The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

Effect of Variation of Mobile Phase Organic Composition:
The sample was analyzed by variation of mobile phase i.e. Methanol: Phosphate Buffer was taken in the ratio and 40:60, 30:70 instead (35:65), remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

III. RESULTS AND DISCUSSION

Analytical Method Development:

Sample & Standard Preparation for the UV-Spectrophotometer Analysis:

25 mg of Darolutamide standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 0.1 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

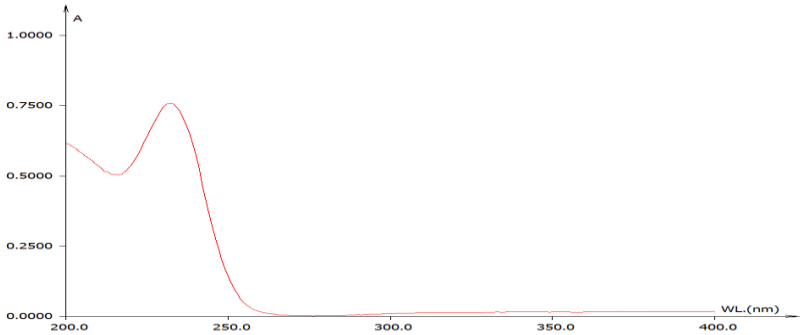

Fig-5.1: UV Spectrum for Darolutamide (235nm)

Observation: While scanning the Darolutamide solution we observed the maxima at 235nm. The UV spectrum has been recorded on T60-LAB INDIA make UV – Vis spectrophotometer model UV-2450.

Optimized Chromatographic Conditions:

Mobile phase ratio : Methanol: Phosphate Buffer (35:65%) v/v
Column : Symmetry ODS C_{18} (4.6mm×250mm, 5µm)
Column temperature : Ambient
Wavelength : 235nm
Flow rate : 1.0 mL/min
Injection volume : 10.0µl
Run time : 8.0 min

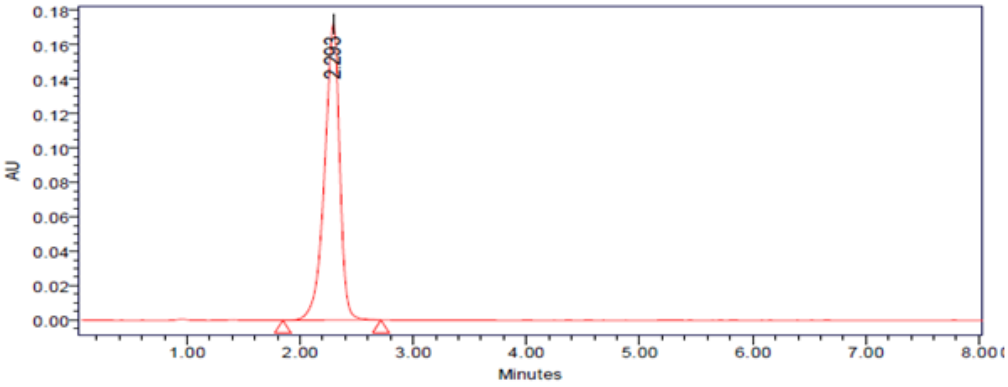

Fig-2: Optimized Chromatographic Condition

Validation of Analytical Method

System Suitability:

The test was carried out by making five replicate injections of a standard solution containing 10µg/ml of Darolutamide and analyzing each solute for their peak area, theoretical plates (N), tailing factor (T) and asymmetric factors (As). The results of System Suitability test[7] were shown in table-3.

Table-3: Results of System Suitability for Darolutamide

S.No.	Peak Name	RT	Area	Height (µV)	USP Plate Count	USP Tailing
1	Darolutamide	2.277	1652847	185647	6589	1.24
2	Darolutamide	2.277	1653658	186254	6587	1.26
3	Darolutamide	2.267	1654521	185475	6584	1.28
4	Darolutamide	2.265	1653564	186594	6582	1.29

5	Darolutamide	2.277	1658745	185684	6895	1.24
Mean			1654667			
Std.Dev.			2355.764			
%RSD			0.142371			

Specificity

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components[8]. Analytical method was tested for specificity to measure accurately quantitatesDarolutamide in drug product.

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

= 99.40%

Conclusion: The % purityof Darolutamide in pharmaceutical dosage form was found to be 99.40%[9].

Linearity: The linearity of the developed method was evaluated by processing five different concentration levels of Darolutamideover the concentration of 6 to 14µg/mL. The linearity plots were acquired by plotting peak response (on X-axis) vs. concentration (on Y-axis)[10-11]. The results of the linearity and calibration curve were represented in Table 4, and Fig-3.

Table-4: Data for Linearity of Darolutamide

Concentration (µg/ml)	Average Peak Area
6	1078475
8	1461129
10	1808358
12	2211573
14	2593778

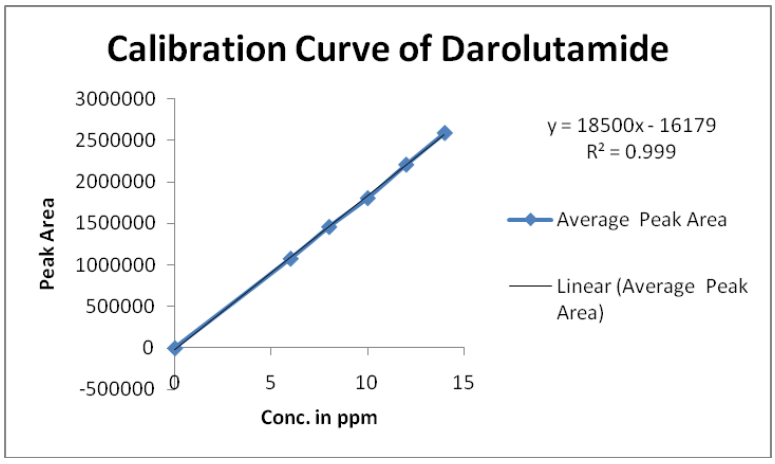

Fig-3: Linearity Curve of Darolutamide

Linearity Plot: The plot of Concentration (x) versus the Average Peak Area (y) data of Darolutamide is a straight line.

Y = mx + c
Slope (m) = 18500
Intercept (c) = 16179
Correlation Coefficient (r) = 0.999

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 0.16179. These values meet the validation criteria.

Precision:
The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions[12].

Repeatability: Obtained Six (6) replicates of 100% accuracy solution as per experimental conditions[13]. Recorded the peak areas and calculated % RSD and results were shown in table-5.

Table-5: Results of Repeatability for Darolutamide:

S. No.	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Darolutamide	2.293	1658954	186958	1.26	6785
2	Darolutamide	2.276	1658745	187548	1.27	6854
3	Darolutamide	2.286	1659865	189854	1.26	6852
4	Darolutamide	2.277	1653254	186985	1.25	6784
5	Darolutamide	2.28	1654781	189542	1.24	6895
6	Darolutamide	2.293	1661324	187586	1.28	6965
Mean			1657821			
Std. Dev			3120.433			
%RSD			0.188225			

Intermediate Precision[14]:
Analyst1:
Table-6: Results of Intermediate PrecisionAnalyst 1 for Darolutamide

S.No.	PeakName	RT	Area (µV*sec)	Height (µV)	USP PlateCount	USPTailing
1	Darolutamide	2.274	1678541	186589	6587	1.26
2	Darolutamide	2.258	1685985	186598	6321	1.26
3	Darolutamide	2.267	1685745	186985	6385	1.25
4	Darolutamide	2.27	1685987	187854	6580	1.26
5	Darolutamide	2.264	1698526	187549	6721	1.27
6	Darolutamide	2.265	1685943	186598	6637	1.26
Mean			1686788			
Std.Dev.			6463.466			
%RSD			0.383182			

Analyst 2:
Table-7: Results of Intermediate Precision Analyst 2 for Darolutamide

S.No.	PeakName	RT	Area (µV*sec)	Height (µV)	USPPlate count	USPTailing
1	Darolutamide	2.277	1665847	167481	6854	1.25
2	Darolutamide	2.255	1658989	167854	6785	1.26
3	Darolutamide	2.265	1659845	167895	6854	1.24
4	Darolutamide	2.255	1665964	167854	6895	1.26
5	Darolutamide	2.253	1659863	168585	6459	1.25

6	Darolutamide	2.252	1665986	167859	6456	1.26
Mean			1662749			
Std.Dev.			3501.766			
%RSD			0.210601			

Accuracy

Method accuracy[15] was estimated at three variable concentrations of 50%, 100%, and 150% levels by spiking the known amount of the drug analytes. The % recovery[16-18]at each level was calculated, and the findings were represented in Table 8.

Table-8: The Accuracy Results for Darolutamide

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	109068.3	5	5.021	100.420%	100.72%
100%	202187	10	10.054	100.540%	
150%	297032.3	15	15.181	101.206%	

Limit of Detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value[19].

$$\mathbf{LOD = 3.3 \times \sigma / s}$$

Where

σ = Standard deviation[20] of the response

S = Slope of the calibration curve

Result:

= 0.95µg/ml

Quantitation Limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined[21].

$$\mathbf{LOQ = 10 \times \sigma / S}$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve[22]

Result:

= 2.9µg/ml

Robustness:

The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Darolutamide[23]. The method is robust only in less flow condition. The standard of Darolutamide was injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count[24].

Table-9: Results for Robustness of Darolutamide

Parameter Used for Sample Analysis	Peak Area	Retention Time	Theoretical Plates	Tailing Factor

Actual Flow rate of 1.0 mL/min	1658242	2.312	6569	1.24
Less Flow rate of 0.9 mL/min	1854215	2.458	6865	1.35
More Flow rate of 1.1 mL/min	1758468	2.032	6254	1.32

Stability Studies

The specificity of the method can be demonstrated by applying stress conditions using acid, alkaline, peroxide, thermal, UV, water degradations. The sample was exposed to these conditions the main peak of the drug was studied for peak purity that indicating the method effectively separated the degradation products from the pure active ingredient[25-26]. The results of Forced Degradation Studiesfor Darolutamide were shown in Table-10.

Table-10: Results of Forced Degradation Studies for Darolutamide

S.No.	Stress Condition	Peak Area	% of Degraded Amount	% of Active Amount	Total % of Amount
1	Standard	1658242	0	100%	100%
2	Acidic	1331734.15	19.69	80.31	100%
3	Basic	1594233.85	3.86	96.14	100%
4	Oxidative	1394747.34	15.89	84.11	100%
5	Thermal	1575827.37	4.97	95.03	100%
6	Photolytic	1345331.73	18.87	81.13	100%
7	Water	1360090.08	17.98	82.02	100%

CONCLUSION

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 235nm and the peak purity was excellent. Injection volume was selected to be 10µl which gave a good peak area. The column used for study was Symmetry ODS C_{18} (4.6mm×250mm, 5µm) because it was giving good peak. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time. Mobile phase is Methanol: Phosphate Buffer pH-3.6 in the ratio of 35:65% v/v was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study. Methanol was selected because of maximum extraction sonication time was fixed to be 10.0min at which all the drug particles were completely soluble and showed good recovery. Run time was selected to be 8min because analyze gave peak around 2.293min and also to reduce the total run time. The percent recovery was found to be 98.0-102% was linear and precise over the same range. Both system and method precision was found to be accurate and well within range. The analytical method was found linearity over the range of 6-14ppm of the Darolutamide target concentration. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

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