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DEVELOPMENT, VALIDATION AND STABILITY INDICATING STUDIES OF A NOVEL ROBUST ANALYTICAL METHOD FOR ANTI-NEOPLASTIC AGENT ESTIMATION USING LIQUID CHROMATOGRAPHY

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ABSTRACT

For the detection of enasidenib, an innovative reversed phase high performance liquid chromatography approach that is simple, fast, accurate, and selective was created and verified. The Symmetry ODS C18 (4.6 x 150 mm, 5 µm) column was used to make the separation. At a wavelength of 272 nm, the mobile phase utilized included ethanol: water adjusted with an orthophosphoric acid solution in a 50:50% v/v ratio in isocratic mode. For the determination

of Enasidenib in bulk and its injected sample volume, the mobile-phase flow rate and the technique that was developed and validated were 1 ml/min and 10 µl, respectively. Enasidenib was shown to have a retention time of 2.8 ±0.2 minutes. Over a concentration range of 20 to 100µg/ml of Enasidenib, a solid linear interaction (r=0.999) was found. Enasidenib's limit of quantification (LOQ) and limit of detection (LOD) were determined to be 6.35 µg/ml and 2.6 µg/ml, respectively. The degree of recovery was found to be between 98 and 102%. The precision study's relative standard deviation was less than 2%. Enasidenib in bulk and marketed pharmaceutical dosage forms may be estimated using the devised approach since it is quick, easy, exact, specific, and accurate. The drug product was subjected to hydrolysis (acid and base hydrolysis), H2O2, thermal degradation, and light degradation during force degradation. Within the specified conditions, the percentage of deterioration for both Enasidenib was determined to be between 10 and 20%. The suggested techniques, which may be applied to the simultaneous estimate of Enasidenib in tablet dose form, were simple, precise, and cost-effective.

Keywords: Enasidenib, RP-HPLC, stability indicating study and ICH Q2 (R1) Guidelines.

1. INTRODUCTION:

Bone marrow hematopoietic stem cells are affected by acute myeloid leukemia (AML) [1]. Inhibition of bone marrow hematopoiesis and the buildup of immature myeloid cells in the bone marrow were the usual characteristics of AML [2]. For example, anemia, perforation, infection, fever, organ of infiltration, and so forth were clinical signs of AML. For example, the illness was severe, the prognosis was risky, and it was frequently fatal [3]. For example, small molecule inhibitors, intense chemotherapy, and non-intensive chemotherapy are now the most common therapeutic approaches for AML [4]. Nevertheless, the overall impact of these medications was poor, necessitating the development of new disease-treating techniques. On August 1, 2017, the FDA in the USA authorized Enasidenib (Figure.1), a small molecule inhibitor of isocitrate dehydrogenase-2 (IDH2), for the treatment of patients with recurrent or refractory AML with IDH2 mutations [5,6]. Enasidenib's half-life, for example, was around 137 hours adhering to oral administration of 100 mg [7]. A rapid, simple, and precise RP-HPLC technique was suggested in this paper.

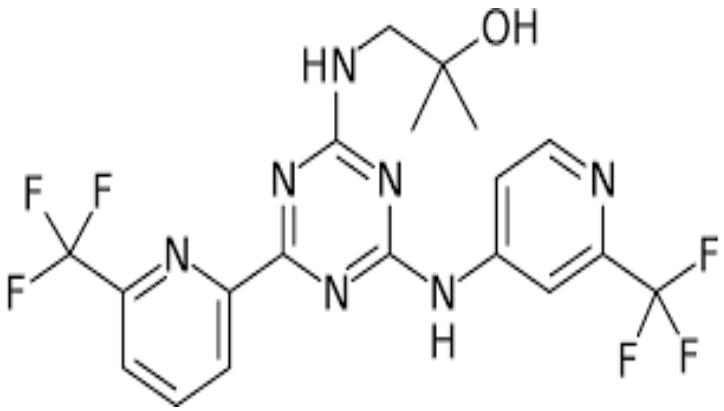


Fig. 1: Chemical Structure of Enasidenib

1.1. DRUG PROFILE

Table .1: Drug Profile

Drug	Enasidenib
Synonym	AG-221 enasidenib Idhifa
IUPAC name	2-methyl-1-[4-[6-(trifluoromethyl)pyridin-2-yl]-6-[2-(trifluoromethyl) pyridin-4-yl] amino]-1,3,5-triazin-2-yl] amino]propan-2-ol
Molecular formula	C19H17F6N7O
Molecular weight	473.4 g/mol
Category	Anti neoplastic agent.
Melting point	216°C
pka	-0.68
Solubility	Practically insoluble (solubility less than equal to 74mlg/ml) in aqueous solution across physiological PH range
Log P	3.5

2. MATERIAL AND METHOD

Instruments used

For the experiment WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector HPLC was used. Vericiguat (Pure) was obtained, Water and Methanol for HPLC was procured from Lichrosolv (Merck) and Acetonitrile for HPLC was procured from Merck

Preparation of standard solution:

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

Further pipette 0.6ml of the above Enasidenib stock solutions into a 10ml volumetric flask and dilute up to the mark with Ethanol.

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.

Mobile Phase Optimization:

Initially the mobile phase tried was Acetonitrile: Water and Ethanol: Water with varying proportions. Finally, the mobile phase was optimized to Ethanol: Water in proportion 50:50 v/v respectively.

Optimization of Column:

The method was performed with various columns like C18 column, X- bridge column, Xterra, and C18 column. Symmetry C18 5µm (4.6×150mm) 5µl was found to be ideal as it gave good peak shape and resolution at 1ml/min flow [8].

Optimized chromatogram (standard)

Column : Symmetry ODS C18 (4.6×150mm, 5µm)

Column temperature : 35ºC

Wavelength : 272nm

Mobile phase ratio : Ethanol: water (50:50% v/v)

Flow rate : 1.0ml/min

Injection volume : 3-10 µl

Run time : 2.8 ± 2min

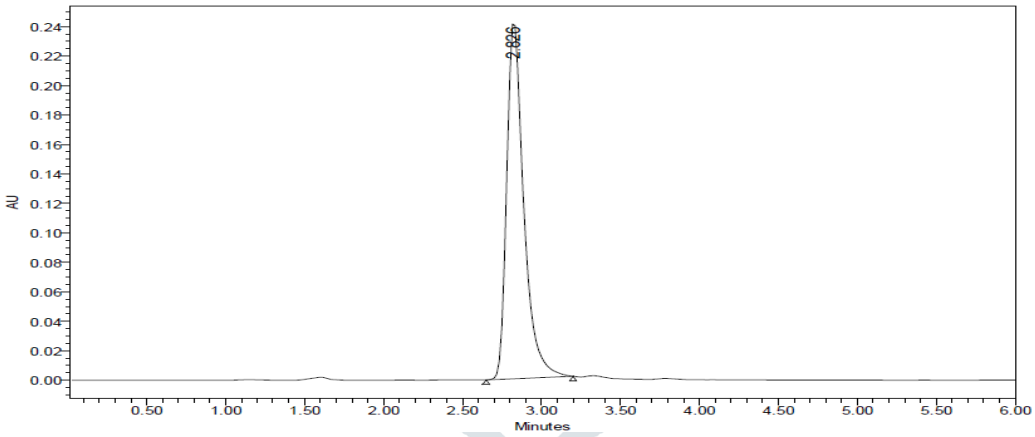


Fig .2: Optimized Chromatogram (Standard)

Table .2: Optimized Chromatogram (Standard)

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Enasidenib	2.826	1825462	132551	1.6	5365

Optimized Chromatogram (Sample)

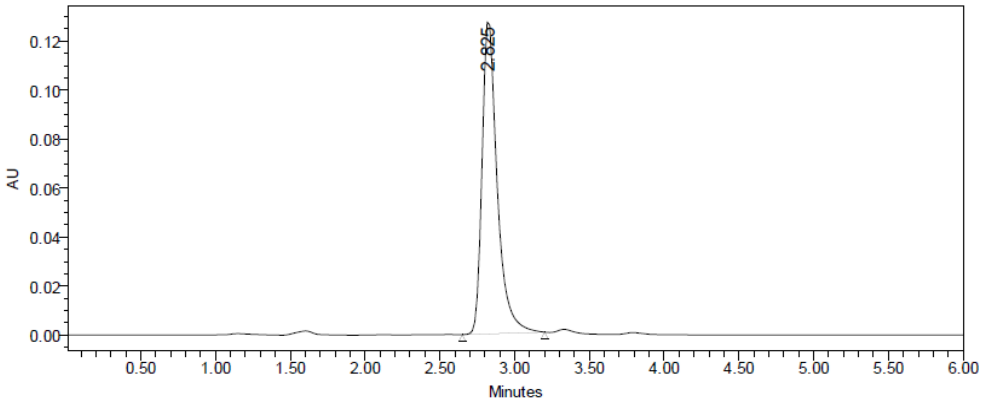


Fig .3: Optimized Chromatogram (Sample)

Table .3: Optimized Chromatogram (Sample)

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Enasidenib	2.825	1836584	138687	1.6	5426

Acceptance criteria:

Theoretical plates must be not less than 2000.Tailing factor must be not more than 2. It was found from above data that all the system suitability parameters for developed method werewithin the limit.

3. VALIDATION

Preparation Of Mobile Phase:

Accurately measured 350 ml (35%) of Ethanol, 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. Mobile phase was used as diluent.[9].

VALIDATION PARAMETERS:

3.1. System suitability

Accurately weigh and transfer 10 mg of Enasidenib 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 0.6ml of the above Enasidenib stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

VALIDATION PARAMETERS:

3.1. System suitability

Accurately weigh and transfer 10 mg of Enasidenib 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 0.6ml of the above Enasidenib stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

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.[10].

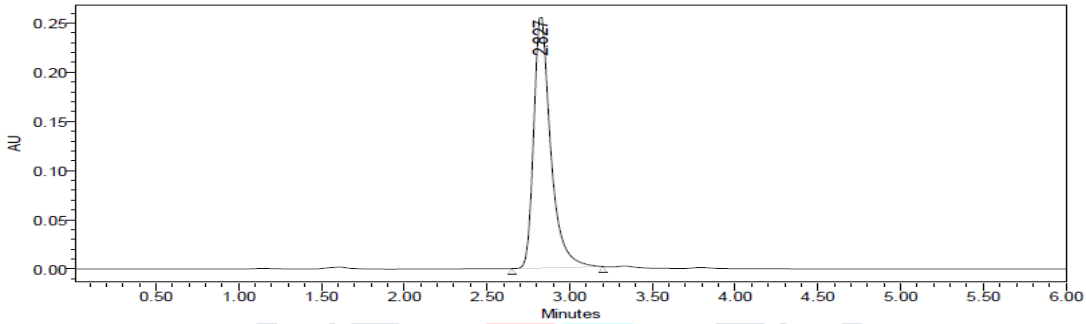


Fig .4: Chromatogram showing good injection

Table .4: Results of system suitability for Enasidenib

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Enasidenib	2.824	1825658	132653	5426	1.6
2	Enasidenib	2.825	1836587	132658	5369	1.5
3	Enasidenib	2.827	1825654	135685	5359	1.6
4	Enasidenib	2.822	1835642	134857	5418	1.6
5	Enasidenib	2.830	1825787	136598	5356	1.5
Mean			1829866			
Std. Dev.			5714.466			
% RSD			0.312289			

Acceptance criteria:

The %RSD of five different sample solutions should not more than 2. The %RSD obtained is within the limit, hence the method is suitable.

3.2. Specificity study of drug:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Enasidenib 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 0.6ml of the above Enasidenib stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of Sample Solution:

Take average weight of the powder and weight 10 mg equivalent weight of Enasidenib 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 0.6ml of Enasidenib 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:

Assay (Standard):

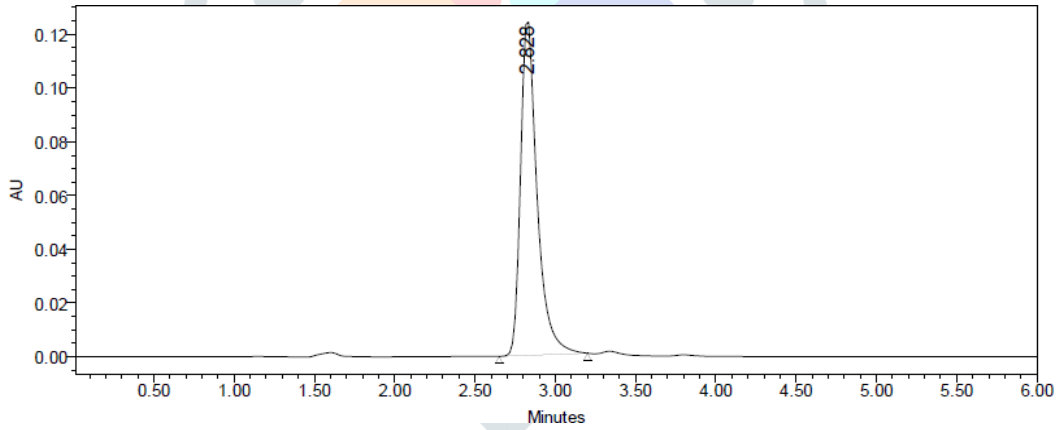


Fig .5: Chromatogram showing assay of standard injection

Table .5: Peak Results for assay standard

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Enasidenib-1	2.828	1836524	134582	1.6	5469
2	Enasidenib-2	2.829	1835648	135629	1.7	5498
3	Enasidenib-3	2.828	1836954	136584	1.6	5568

Assay (Sample):

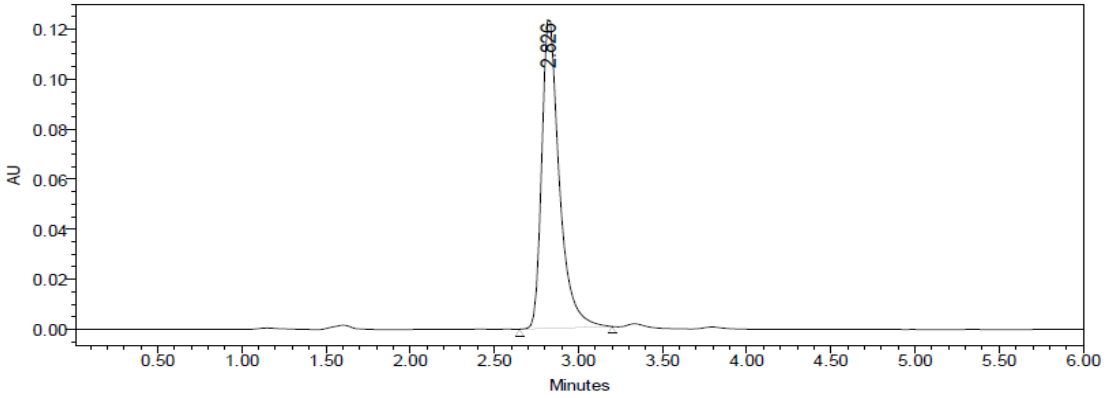


Fig .6: Chromatogram showing assay of sample injection

Table .6: Peak results for Assay sample

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Enasidenib-1	2.826	1826523	134568	1.7	5658
2	Enasidenib-2	2.825	1825475	135698	1.6	5487
3	Enasidenib -3	2.833	1825748	135688	1.6	5698

3.3. Linearity:

Accurately weigh and transfer 10 mg of Enasidenib 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. (preparation of different Stock solution are:20ppm, 40ppm, 60ppm, 80ppm, 100ppm).

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.

Table .7: Linearity data for Enasidenib

Concentration □g/ml	Average Peak Area
20	668748
40	1278875
60	1886598
80	2458644
100	3028547
Correlation coefficient	0.99%

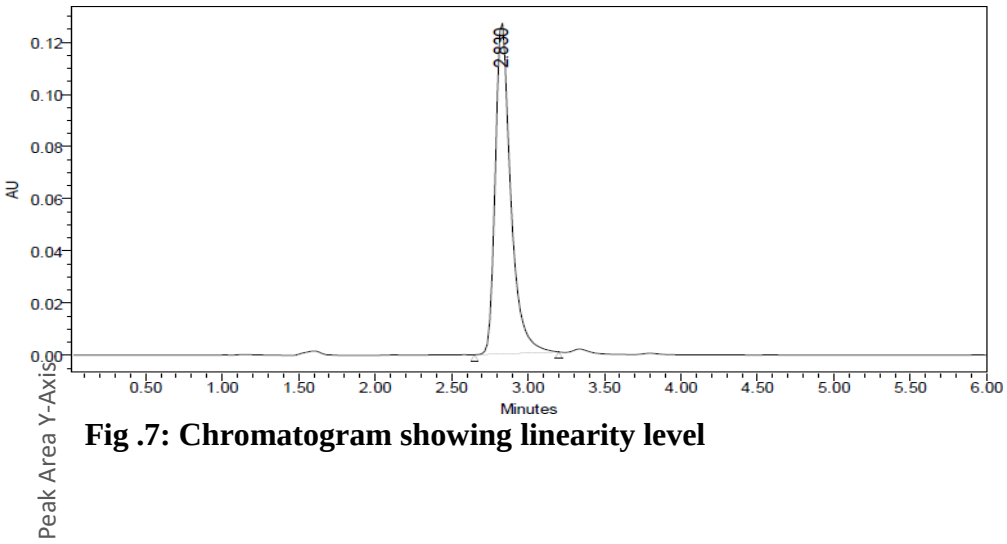


Fig .7: Chromatogram showing linearity level

Linearity plot:

The plot of Concentration (x) versus the Average Peak Area (y) data of Enasidenib is a straightline.

Y = mx + c

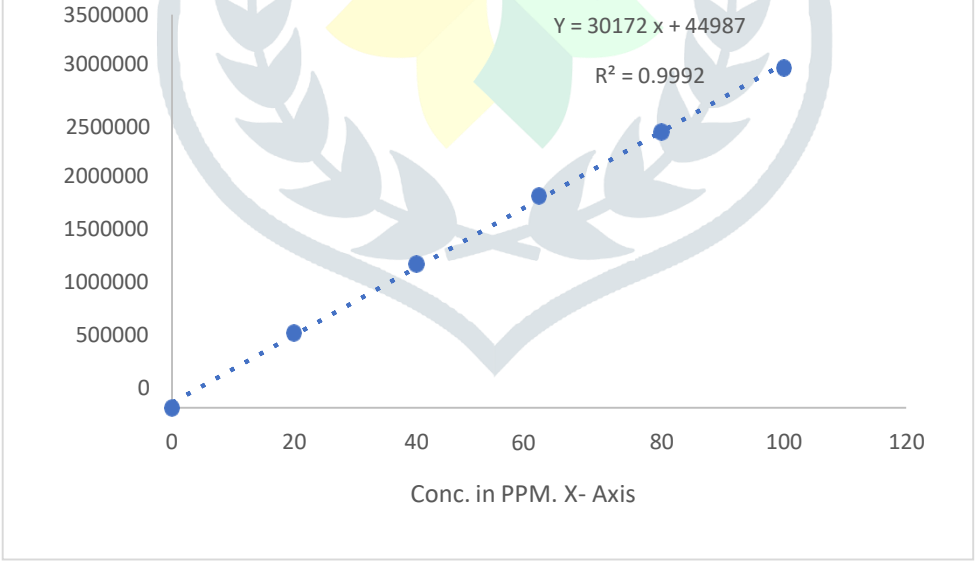
Slope (m) = 30172 Intercept (c) = 44987

Correlation Coefficient (r) = 0.99

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 44987. These values meet thevalidation criteria.

Fig.8: CALIBARTION CURVE FOR ENASIDENIB



3.4. 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.

3.5. Repeatability

Preparation of Enasidenib Product Solution for Precision:

Accurately weigh and transfer 10 mg of Enasidenib 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 0.6ml of the above Enasidenib stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

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.

Obtained Five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table .8: Results of Repeatability for Enasidenib:

S. No	Peak name	Retentiontime	Area(µV*sec)	Height(µV)	USP Plate Count	USP Tailing
1	Enasidenib	2.824	1825463	133526	5426	1.6
2	Enasidenib	2.827	1825685	132564	5369	1.7
3	Enasidenib	2.833	1825426	133254	5428	1.6
4	Enasidenib	2.833	1835687	132546	5385	1.6
5	Enasidenib	2.836	1825642	132658	5364	1.6
Mean			1827581			
Std.dev			4532.982			
%RSD			0.248032			

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:

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.

Table .9: Results of Intermediate precision analyst for Enasidenib

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	Plate Count	USPTailing
1	Enasidenib	2.823	1836524	133658	469	1.6
2	Enasidenib	2.827	1836875	133695	487	1.7
3	Enasidenib	2.828	1836958	133693	436	1.6
4	Enasidenib	2.828	1836597	134568	498	1.6
5	Enasidenib	2.825	1845689	134598	426	1.6
6	Enasidenib	2.822	1845784	133659	468	1.7
Mean			1839737			
Std. Dev.			4649.5042			
% RSD			0.253%			

3.6. Accuracy:

For preparation of 50% Standard stock solution: Accurately weigh and transfer 10 mg of Enasidenib 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 0.3ml of the above Enasidenib 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 Enasidenib 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 0.6ml of the above Enasidenib 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 Enasidenib 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 0.9ml of the above Enasidenib 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 Enasidenib and calculate the individual recovery and mean recovery values.

Table .10: Results of Accuracy for concentration-50%, 100% and 150%.

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Enasidenib -1	2.836	952654	131265	1.2	4896
	Enasidenib -2	2.838	951658	130269	1.3	4798
	Enasidenib -3	2.853	952364	131258	1.2	4674
2	Enasidenib -1	2.826	1862587	132658	1.7	5469
	Enasidenib -2	2.830	1860598	133265	1.6	5396
	Enasidenib -3	2.822	1865984	132698	1.7	5475
3	Enasidenib -1	2.831	2765847	165325	1.9	6125
	Enasidenib -2	2.835	2768542	166532	1.8	6239
	Enasidenib-3	2.839	2759898	165878	1.9	6126

Table .11: The accuracy results for Enasidenib

% Concentration (at specification Level)	Area	Amount Added (ppm)	AmountFound (ppm)	% Recovery	Mean Recovery
50%	952225.3	30	30.068	100.226%	100.27%
100%	1863056	60	60.256	100.426%	
150%	2764762	90	90.142	100.157%	

Acceptance Criteria:

The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

3.7. 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 Enasidenib 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 0.6ml of the above Enasidenib 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 0.8ml/min and 0.9ml/min instead of 1.0ml/min, remainingconditionsare same. 10µl of the above sample was injected and chromatograms were recorded.

Table 25: Results for Robustness

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0mL/min	1825462	2.826	5365	1.6
Less Flow rate of 0.8mL/min	1818987	3.13	5126.3	1.7
More Flow rate of 1.0mL/min	1812658	2.589	5168.4	1.6
More Organic phase	1815897	2.514	5268.9	1.6
Less Organic phase	1805896	3.344	5264.4	1.7

Acceptance criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

Effect of Variation of mobile phase organic composition:

The sample was analyzed by variation of mobile phase i.e. Ethanol: Water was taken in the ratio and 45:55, 55:45 instead of 50:50, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

3.8. Limit of detection for Enasidenib

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

LOD= 3.3 × σ / s

Where

σ = Standard deviation of the responseS = Slope of the calibration curve **Result:**= 2.6µg/ml

3.9. Quantitation limit of Enasidenib

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

LOQ=10× σ/S

Where

σ = Standard deviation of the responseS = Slope of the calibration curve **Result:**= 6.35µg/ml.STABILITY STUDIES:

The stability of the developed method was established by performing forced degradation studies of the drug in the presence of acid, alkali, hydrogen peroxide, temperature, light.

3.1. Acid degradation **Degradation under acidic condition was evaluated by treating 1 ml of standard stock solution of Enasidenib with 1 ml of 2N HCl and refluxed for 30 min at 60 ± 2 °C. The resulting solution was diluted to 10 ml with the diluent.**

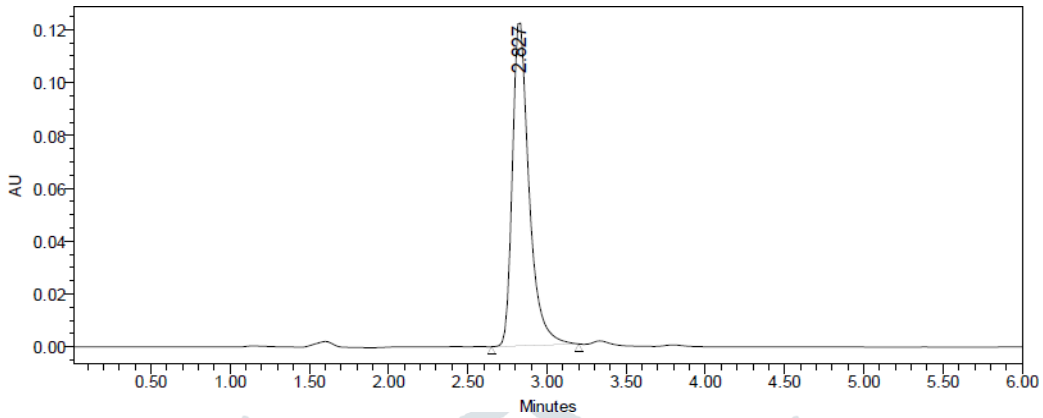


Fig .10: Chromatogram of acid degradation solution

3.2. Alkali degradation

Under alkaline conditions, degradation was studied by refluxing 1 ml of standard stock solution of Enasidenib with 1 ml of 2N NaOH for 30 min at 60 ± 2 °C. The stressed solution was made up to 10 ml with the diluent.

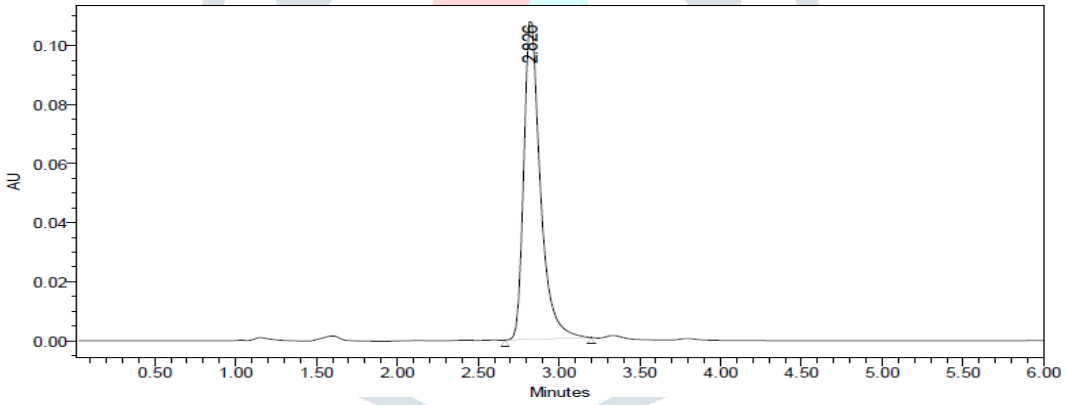


Fig .11: Chromatogram of alkaline degradation solution

3.3. **Oxidative degradation**About 1 ml of standard stock solution of Enasidenib was subjected to oxidative degradation by refluxing with 20% v/v H2O2 in a 10ml volumetric flask for 30 min at 60 ± 2 °C and made up with the diluent.

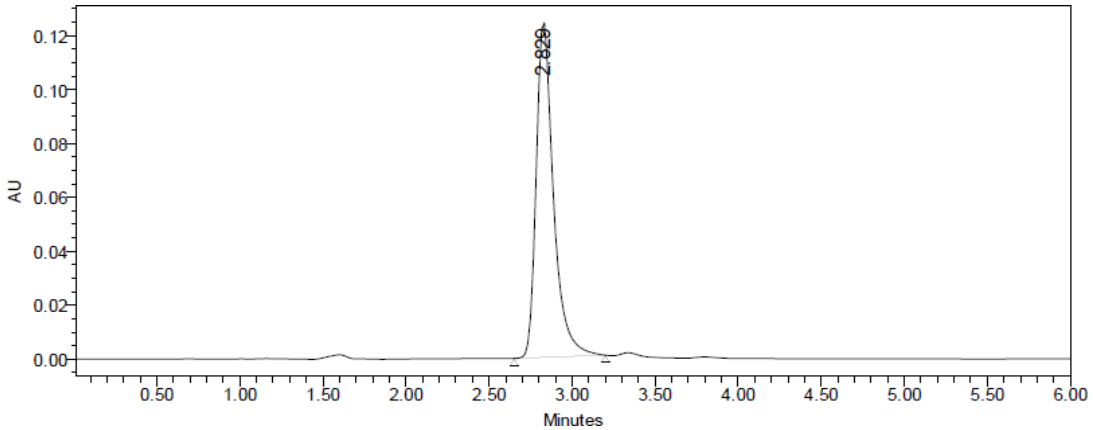


Fig .12: Chromatogram of oxidative degradation solution

3.4. Thermal degradation

Thermal stability of the drugs was evaluated by placing the standard stock solution in the oven at105 ± 2 °C for 6 h. About 1 ml of the stressed solution was diluted to 10 ml with the diluent.

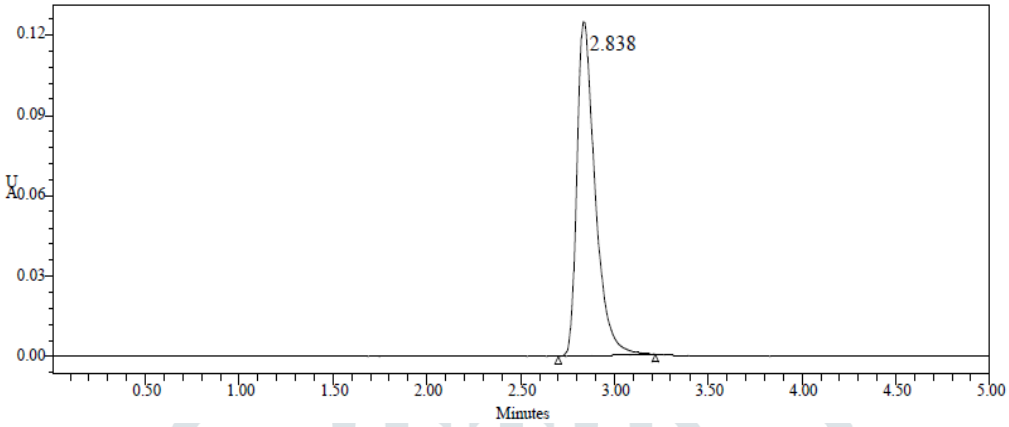


Fig .13: Chromatogram of thermal degradation solution

3.5. Photolytic degradation

Photolytic degradation was studied by exposing the standard solution of Enasidenib to sun lightfor 7 days. The resulting stressed solution was diluted to 10 ml with the diluent.

About 10 µl of each of the solutions exposed to different stress conditions were injected separatelyinto the column, and the chromatograms were recorded to evaluate the stability of the drugs.

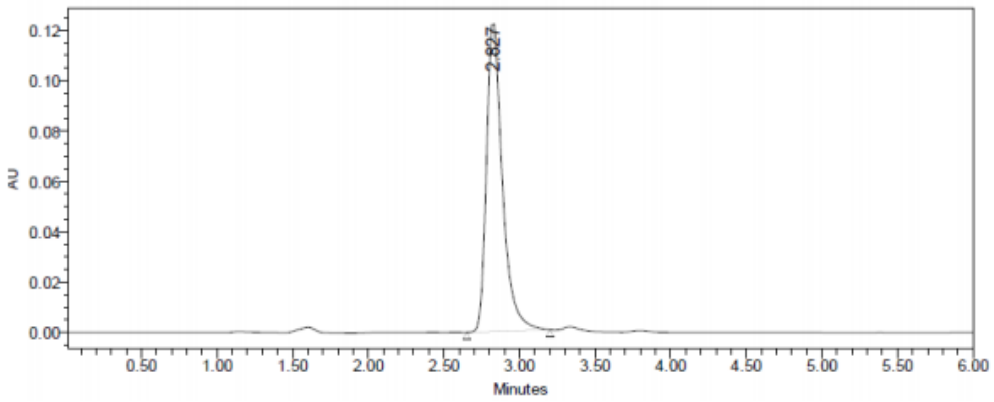


Fig .14: chromatogram for photolytic degradation

From the forced degradation conditions, it was observed that degradation of Enasidenib. As per ICH guidelines, the limit of acceptable forced degradation is less than 20%. In the proposed method, the degradation of Enasidenib was less than 20%, which represents the stability-indicating method.

Table 13: Summary of forced degradation studies of Enasidenib

Stressor	% degradation of Enasidenib
Acid	0.376

Base/Alkaline	1.31
Oxidative	0.214
Photolytic	0. 575
Thermal	0.256

4. CONCLUSION

A straightforward, sensitive, accurate, and exact RP-HPLC technique was created for the quantitative quantification of enasidenib in the current study. Several characteristics were studied in order to design the analytical approach. First, it was discovered that the peak purity was excellent and the highest absorbance was at 272 nm. A satisfactory peak area was obtained with an injection volume of 10µl. Symmetry ODS C18 (4.6×150mm, 5µm) was the column chosen for the investigation since it produced a nice peak. The temperature of 40 oC was determined to be appropriate for the kind of prescription drugs solution. A excellent peak area and a sufficient retention time frame led to the flow rate being set at 1.0 ml/min. Enasidenib dissolved somewhat in water, DMSO, and methanol. The ethanol:water (50:50% v/v) mobile phase was fixed because of its well-symmetrical peak. Thus, the suggested study made use of this mobile phase. Because the maximal extraction sonication duration was set at 15 minutes, at which point all of the drug particles were fully soluble and indicated excellent recovery, ethanol was chosen. Run time was selected to be 7min because analyze gave peak around 2.826 and also to reduce the total run time. Since diluted samples are employed instantly without any prior chemical derivatization or purification processes, this approach proved straightforward. The approach was proven to be accurate, and the ratio of RSD values were within 2. The RP-HPLC method's results, as shown in the tables, were encouraging. The RP-HPLC method outperforms the spectrophotometric approaches in terms of sensitivity, accuracy, and precision. Enasidenib in pharmaceutical dose forms and bulk drugs may be routinely determined using this technology. Results demonstrated that the approach was appropriate for examining the stability of enasidenib under a range of forced degradation scenarios, including oxidation, photolytic degradation, dry heat, acid, and base. In summary, the technique isolates the medications from the byproducts of their breakdown; it may be used to analyze the stability of their tablet dosage form. Still, no characterisation of the degradation products was done.

AUTHORS CONTRIBUTION
all the authors was equally contributed.

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CONFLICTS OF INTERESTS
Declared none.

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