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RP-HPLC ANLAYTICAL METHOD DEVELOPMENT, VALIDATION AND ESTIMATION OF ANAGRELIDE HYDROCHLORIDE IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

A simple, fast and reproducible reverse phase liquid chromatography (RP-HPLC) method was developed for the determination of Anagrelide Hydrochloride in pharmaceutical dosage form. The method was developed Kromasil C18 column (150 x 4.6 mm; 5µm) and a diode array detector (Model 2996) was employed for the study **Mobile phase**: phosphate buffer (pH 2.5) - acetonitrile (75:25 v/v) with isocratic elution at a flow rate of 1 mL/min. System Suitability test were performed for the assurance of quality performance of method. The drug was subjected to accelerated degradation for photolytic, hydrolytic, thermal, oxidative conditions. The retention time of Anagrelide Hydrochloride was found to be 4.8 min. The method was validated for accuracy, precision, specificity, linearity, limit of detection, limit of quantitation and robustness as per ICH guidelines. All the parameters were within limits. The proposed method gave good resolution of Anagrelide Hydrochloride and its degradation products. The developed method can be used for the routine quality control analysis.

Keywords*: RP-HPLC, Anagrelide, Validation, ICH.*

1. INTRODUCTION

Anagrelide Hydrochloride [1] Monohydrate 6,7-dichloro-1H,2H,3H,5H-imidazolidino[2,1-b]quinazolin-2-one C10H7Cl2N3O. HCl.H2O Anagrelide HCl [2,3] inhibits phosphodiesterase III which is found in thrombocytes and cause that few analytical methods available for determination of anagrelide [4-8] by RP-HPLC, LC-MS, UV spectrophotometry. The aim of the present work was the development of a RP-HPLC method for the estimation of Anagrelide Hydrochloride in a pharmaceutical dosage form.

2. MATERIALS AND METHODS

A Waters Alliance liquid chromatography (Model 2695) equipped with a Kromasil C18 column (150 x 4.6 mm; 5µm) and a diode array detector (Model 2996) was employed for the study. Sample injections were done with an automatic injector. Empower2 software was used for data handling. Solubility of the substances was enhanced by sonication on an ultrasonicator (Ultrasonics 3.51). Weighing of the substances in the experiments was done on a Sartorius balance (Model CPA225D).

Drugs, chemicals and solvents:

A pure sample of anagrelide (99.4% purity) obtained from Aarey Drugs & Pharmaceuticals Ltd, Boisar, Maharashtra was used as the reference standard. The commercial capsule formulation of anagrelide „Agrylin (1.0 mg)" manufactured by Shire Manufacturing Inc. was used in this study. Potasium dihydrogen orthophosphate, orthophosphoric acid, sodium hydroxide, HPLC grade acetonitrile and methanol were purchased from Rankem Fine Chemicals Ltd., Mumbai. HPLC grade water was prepared by using Millipore Milli-Q system.

Preparation of the buffer solution (pH 2.5):

6.8 g of potassium dihydrogen orthophosphate was transferred into a beaker containing 800 mL of water. The contents were mixed well and the volume was made up to 1000 mL with water. The pH of the solution was adjusted to 2.5 with orthophosphoric acid. The solution was then filtered through a 0.45µ membrane filter.

Preparation of the mobile phase:

A mixture of the above phosphate buffer (pH2.5) and acetonitrile in the ratio of 75:25 v/v was prepared by mixing 750 mL of the buffer with 250 mL of acetonitrile in a one liter flask. The contents were degassed in an ultrasonic bath for 5 min, and then filtered through a 0.45 µ membrane filter. This mixture was used as the mobile phase in the chromatography.

Stock and working standard solution of anagrelide:

About 10 mg of the reference sample of anagrelide was accurately weighed and transferred into a 10 mL volumetric flask. To this, 2.0 mL of 0.1N

Sodium hydroxide solution was added and sonicated for 5 minutes to dissolve the drug. The volume was made up with methanol and mixed well. This solution was used as the stock solution.

The working standard solution was prepared by transferring 1.0 mL of the stock solution into a 10 mL volumetric flask and diluting to volume with the diluent. This solution (100 µg/mL) was used as the working standard solution of anagrelide.

Preparation of the diluents:

A mixture of the phosphate buffer (pH 2.5) and acetonitrile in the ratio of 50:50 v/v was used as the diluent.

3. OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS AND METHOD DEVELOPMENT

After a number of trials a mixture of phosphate buffer (pH 2.5) and acetonitrile in 75:25 v/v ratio was selected as the mobile phase for separation of anagrelide. The solvent was pumped at a flow rate of 1.0 mL/min. The injection volume was 10 µL and the column temperature was maintained at 25°C. The detector wavelength was set at 250 nm for monitoring the analytes. Prior to injection of the drug solution, the column was equilibrated for atleast 20 min by pumping the mobile phase through it. Typical chromatogram of the working standard solution of anagrelide is shown in Fig. 2.2.

Table 1. Optimized chromatographic conditions of the proposed method

Stationary phase	: Kromasil C18 (150 x 4.6 mm, 5 µm)
Mobile phase	: phosphate buffer (pH 2.5) - acetonitrile (75:25 v/v)
Flow rate	: 1.0 mL/min
Column temperature	: 25°C Injection
volume	: 10 µL
Detection wavelength	: 250 nm
Run time	: 8 min

The retention time (R_t) obtained for anagrelide under the above optimized conditions was **4.818** min.

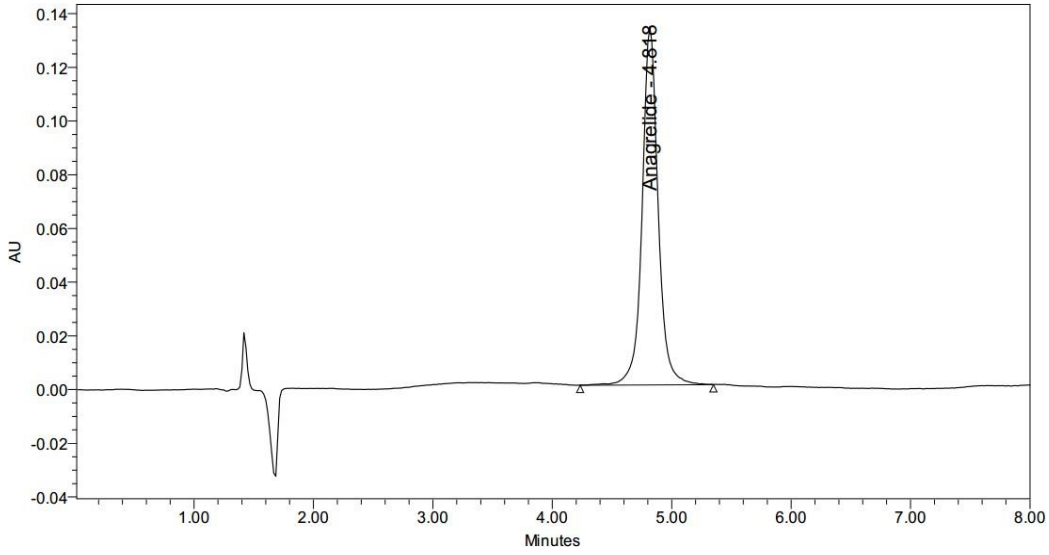

Figure 1 A representative chromatogram of the working standard solution of anagrelide

3.1 VALIDATION OF THE PROPOSED METHOD

Linearity:

Solutions of anagrelide at different concentration levels including the working standard concentration were prepared in the diluent. Ten microlitres of each concentration was injected three times into the HPLC system (n=3). The response was read at 250 nm and the corresponding chromatograms were recorded. From these chromatograms, the mean peak areas at the different concentration levels were calculated and the linearity plot of the mean peak areas over concentration was constructed.

Linearity data for anagrelide is presented in the Table 2. The corresponding plot for anagrelide is depicted in the Fig. 2.

Table 2 Linearity data for anagrelide

Concentration of anagrelide (µg/mL)	Peak area	Mean peak area	SD	%RSD
50	612498	610962.3	1438.595	0.23
	610743			
	609646			
70	859785	860698.3	1149.43	0.13
	860321			
	861989			
	1239846			
	1223570			

100	1219789	1227735	10657.45	0.86
120	1492725	1492404	2314.257	0.15
	1494541			
	1489946			
150	1839412	1848889	11683.09	0.63
	1845312			
	1861942			

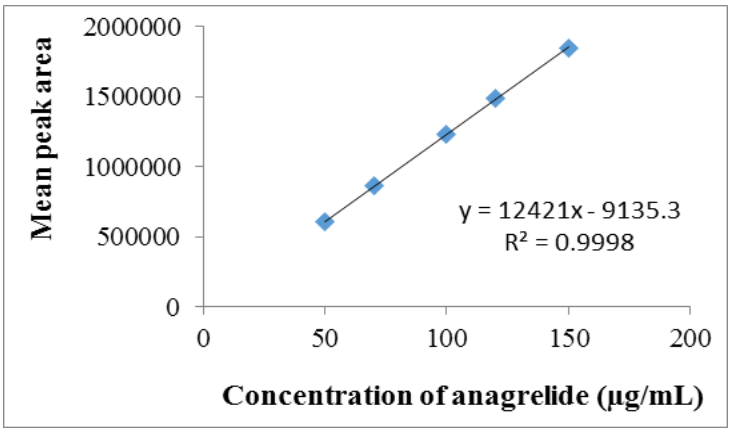

Fig. 2. Linearity plot for anagrelide

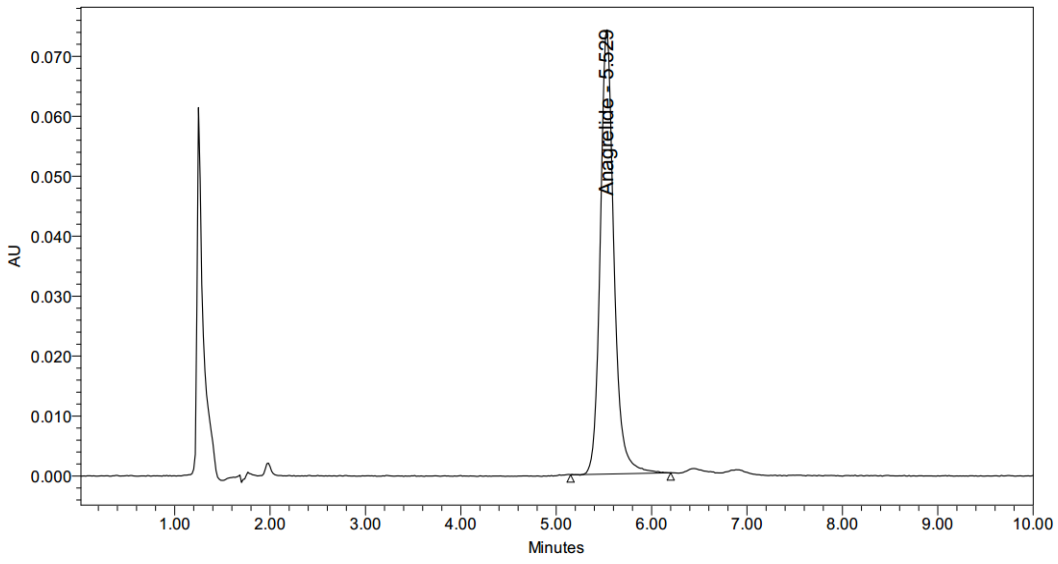

Figure 3.1. A representative chromatogram of 50 µg/mL concentration of anagrelide

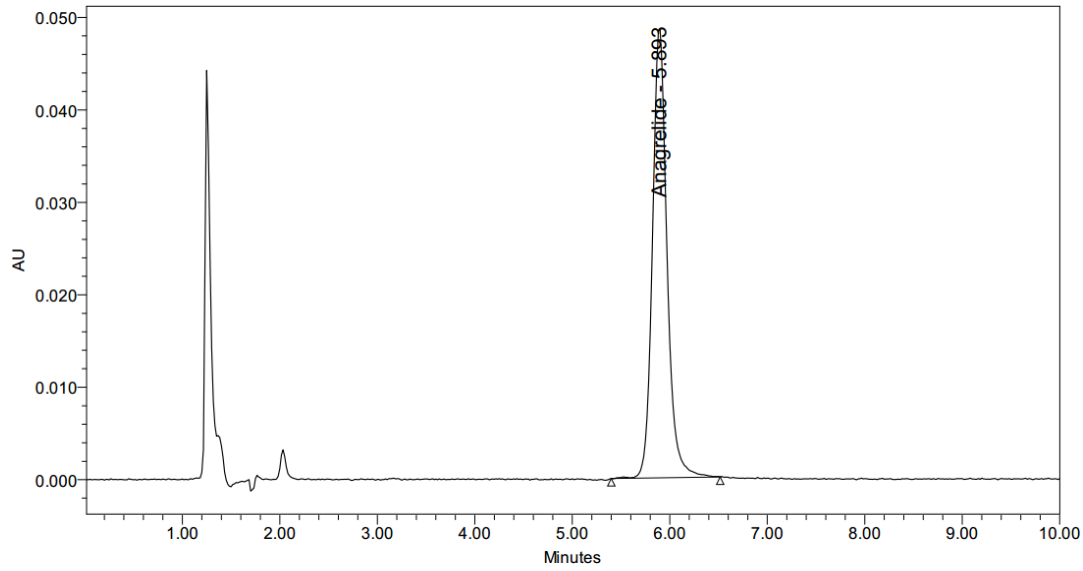

Figure 3. A representative chromatogram of 70 µg/mL concentration of anagrelide

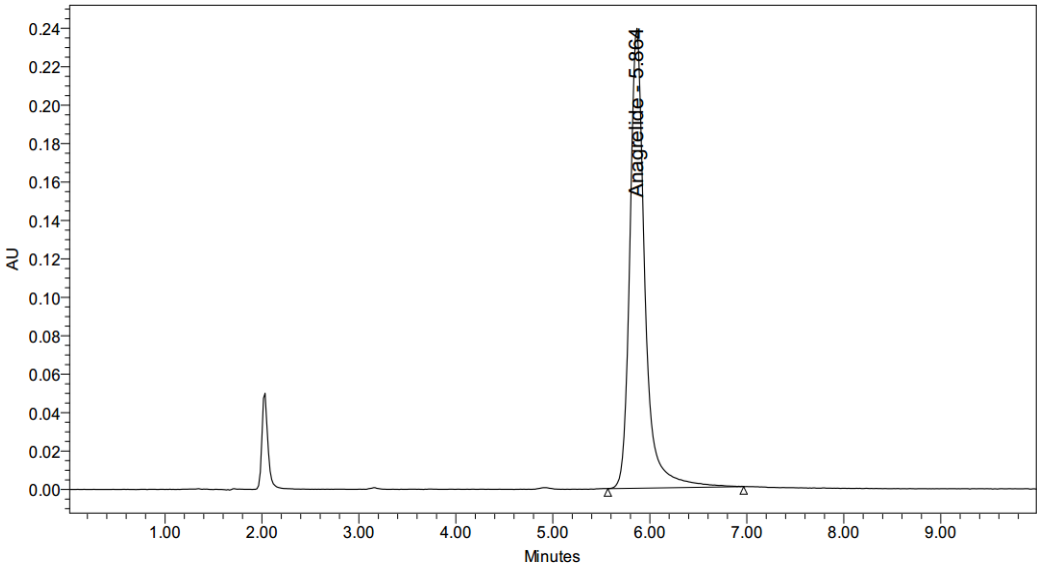

Figure 3. A representative chromatogram of 100 µg/mL concentration of anagrelide

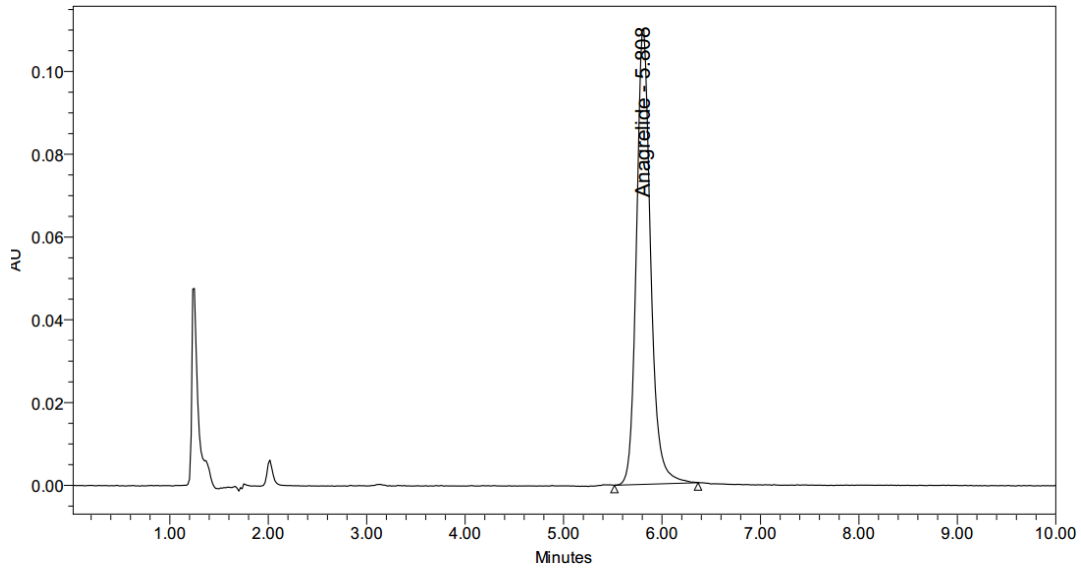

Figure 4. A representative chromatogram of 120 µg/mL concentration of anagrelide

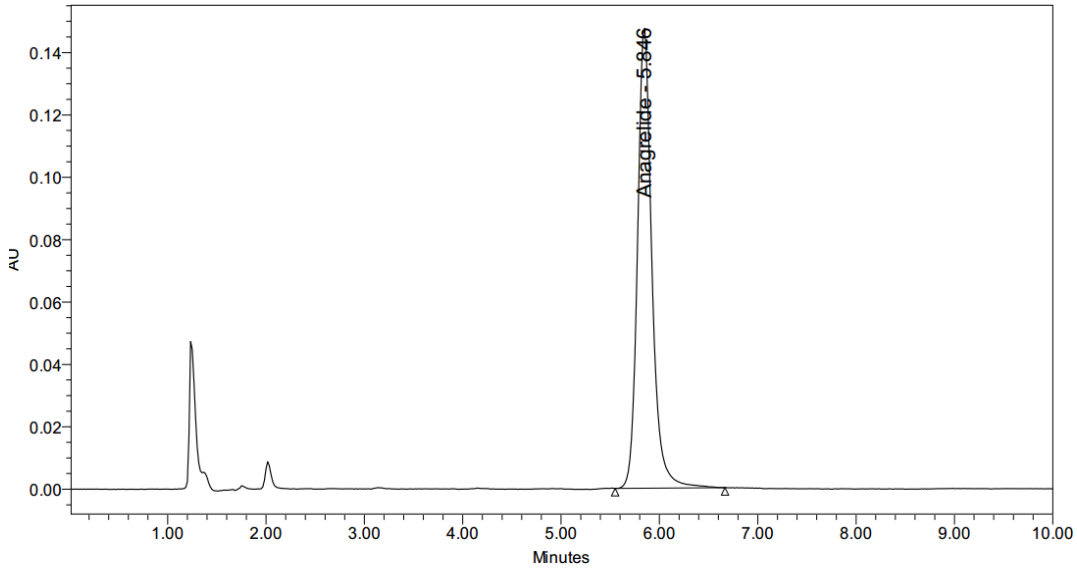

Figure 5. A representative chromatogram of 150 µg/mL concentration of anagrelide

Precision:

Repeatability and intermediate precision were assessed by analyzing standard solutions of anagrelide on the same day (n=6) and on two consecutive days respectively. The results of repeatability and intermediate precision studies are depicted in the Tables 2.4 and 2.5.

Table 3. Repeatability data

S. No.	Peak areas of anagrelide
1	1239846
2	1223570

3	1219789
4	1219985
5	1224567
6	1231256
Average	1226502
SD	7752.927
%RSD	0.63

Table 4. Intermediate precision data

Day	**Average peak areas of anagrelide (n=6)**
1	1220080
2	1235106
Overall average	1227593
SD	10865.18
%RSD	0.88

Accuracy:

The accuracy of the method was determined by analyzing in triplicate known concentrations of the drug corresponding to 50, 100 and 150 % levels of anagrelide (50, 100 and 150 µg/mL). The percent recovery was calculated. The results are incorporated in the Table 2.6.

Table 5. Recovery data of anagrelide

Concentration (µg/mL)	**Peak area**	**Recovery**	**Mean recovery**	**SD of recoveries**	**% recovery**
50	615394	50.00	99.79	0.23	100.00
	614378	49.92			99.83
	612591	49.77			99.54
100	1221473	99.24	100.20	0.94	99.24
	1233651	100.23			100.23
	1244693	101.13			101.13
150	1815748	147.53	98.47	0.47	98.35
	1827314	148.47			98.98
	1810526	147.10			98.07

Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and LOQ were calculated by using residual standard deviation of the response and the slope of the regression line. The LOD and LOQ of anagrelide were found to be 2.035 and 6.168 µg/mL respectively.

Robustness study:

The robustness of the method was determined as per ICH guidelines under three conditions i.e. flow rate, temperature and mobile phase composition. The results obtained by deliberate variation in method conditions are summarized below.

Table 6. Robustness data

Chromatographic condition	Value	Retention time (min)	Tailing factor	Number of theoretical plates
Flow rate (mL/min)	0.9	5.648	1.15	4019
	1.0(O)	4.818	1.12	4203
	1.1	3.614	1.12	4377
Temperature (°C)	23	4.420	1.14	4294
	25(O)	4.818	1.12	4325
	27	4.265	1.11	4385
Mobile phase composition (Phosphate buffer : Acetonitrile (% v/v))	76:24	4.991	1.14	4184
	75:25(O)	4.818	1.12	4357
	74:26	4.729	1.13	4682

(O) – Optimised value.

Specificity of the proposed method:

The specificity of the method was evaluated with regard to interference due to presence of excipients in tablet formulation. The HPLC chromatograms recorded for the drug matrix did not show any interfering peak within retention time ranges. Fig. 2.2 and 2.9 show the representative chromatograms obtained from the analysis of anagrelide from working standard solution and the formulation sample solution. The figures show that the selected drug was clearly separated.

System suitability:

For finding out system suitability, six replicates of the working standard sample were injected and the parameters like peak retention time, tailing factor, number of theoretical plates (N) and HETP of the peak were generated. These results are shown in Table 2.8.

Table 7. System suitability parameters of the proposed method

S. No.	Parameter	Result
1	Retention time (min)	4.818

2	Tailing factor	1.12
3	Theoretical plates	4357
4	HETP	0.03271

Estimation of the drug from capsule dosage forms:

The contents of five capsules of ``Agrylin`` were transferred into a 50 mL volumetric flask. To this, 30 mL of the diluent was added and sonicated for 25 min. Then the volume was made up with the diluent and the contents were mixed well to get a final concentration of 100 µg/mL. This mixture was filtered through a 0.45µ membrane filter (discarding the first few mL of the filtrate).This solution was then chromatographed six times and from the chromatograms obtained, the average drug content in the formulation was calculated. A typical chromatogram obtained from the analysis of Agrylin capsule is shown in the Fig. 6.

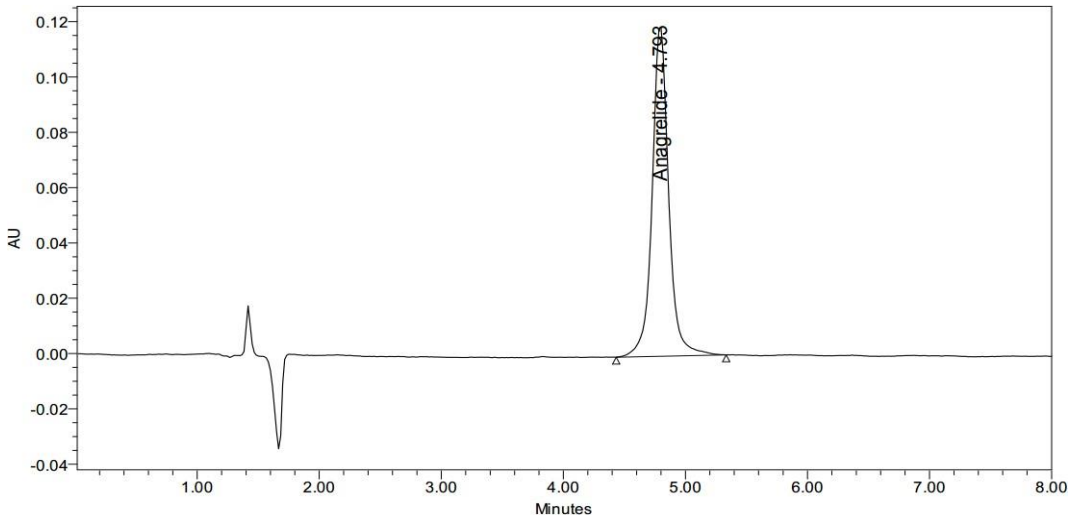

Figure 6. A representative chromatogram of the capsule formulation of anagrelide

Method suitability:

Studies for recovery of anagrelide from its commercial capsule formulations (Agrylin) were carried out by the proposed method and the results are shown in Table 2.9. The values obtained were found to be in good agreement with the labelled amounts. This confirms the suitability of the method for the analysis of anagrelide in capsule dosage form.

Table 2.9 Recovery of the drug from the capsule dosage form Agrylin

Drug	Labelled amount (mg)	Average Amount recovered (mg) (n=6)	% Recovery
Anagrelide	1.0	0.99	99

4. APPLICABILITY OF THE METHOD FOR MONITORING FORCED DEGRADATION STUDIES ON THE DRUG

In order to evaluate the stability of anagrelide and ability of the proposed method to separate anagrelide from its degradation products, anagrelide was subjected to various stress conditions such as acidic condition (2N hydrochloric acid), alkaline condition (2N sodium hydroxide), dry heat

condition (105 ° C for 6 hr), oxidation (20 % w/v of hydrogen peroxide), photolysis (exposure to ultraviolet radiation) and neutral degradation. The chromatograms are presented in Fig.2.10 to 2.16**.** The results are summarized in Table 2.10 and Table 2.11.

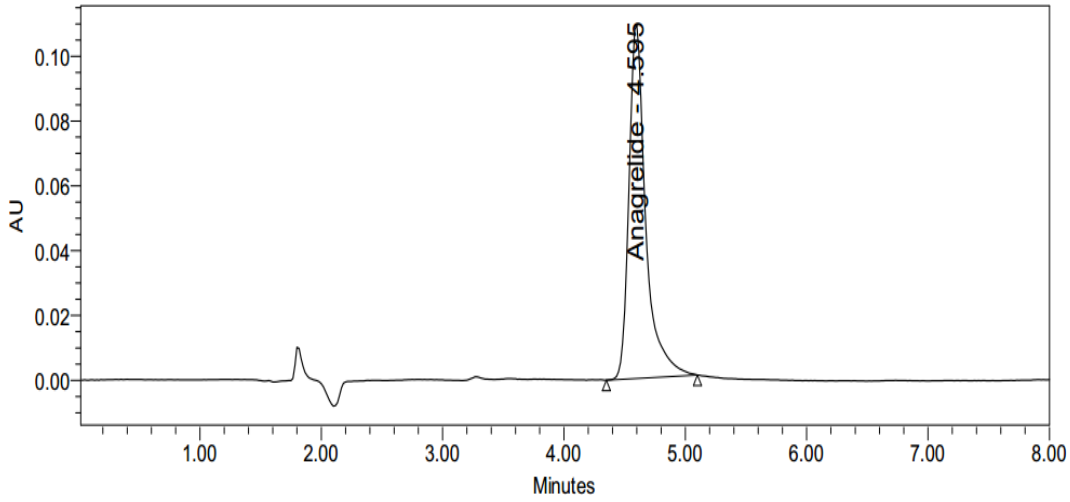

Fig.7. Chromatogram: Standard drug solution of anagrelide.

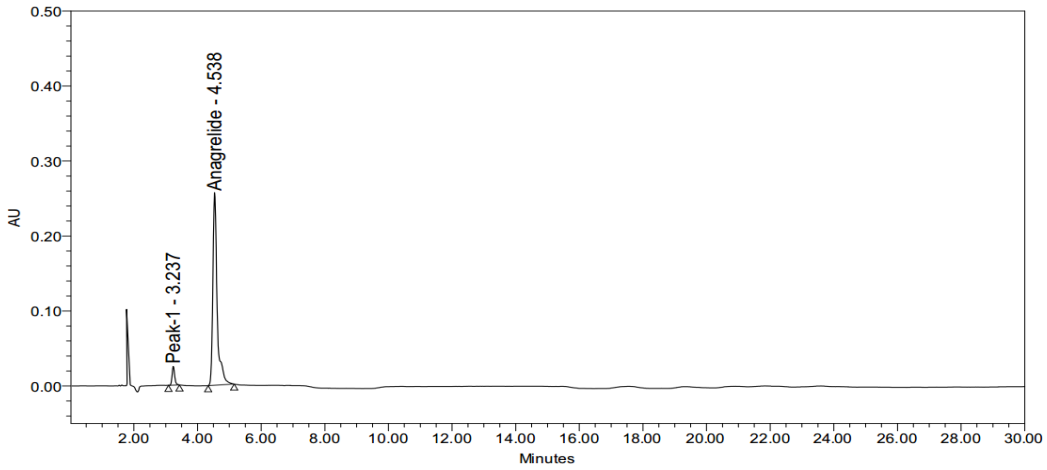

Fig.8. Chromatogram : Acid degradation of anagrelide

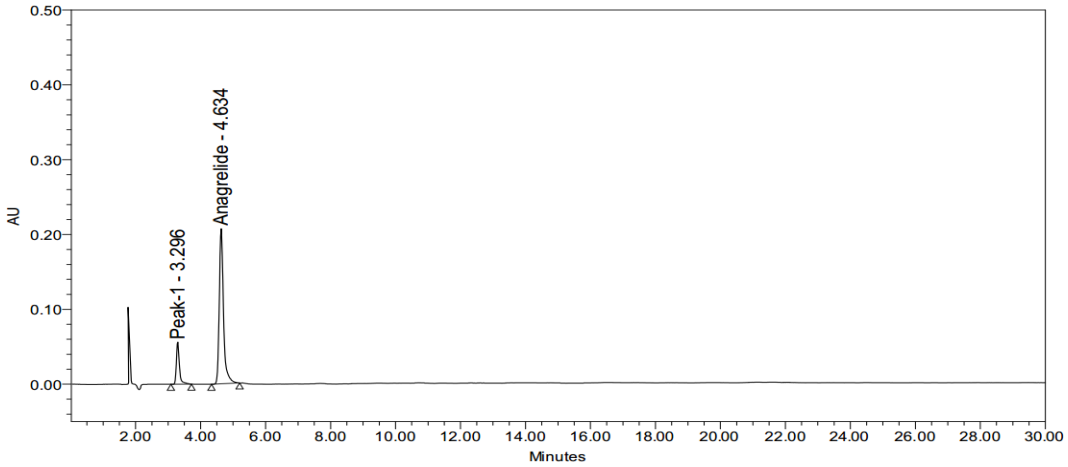

Fig.9. Chromatogram : Base degradation of anagrelide

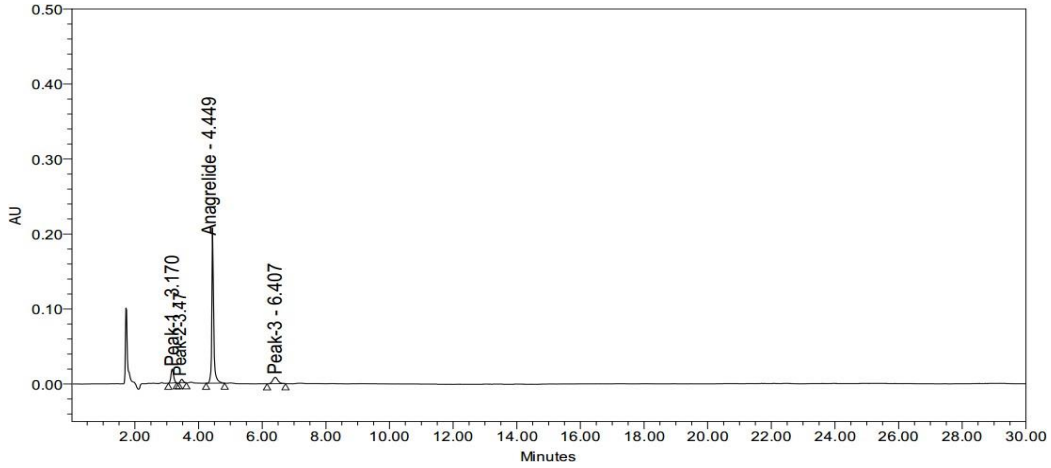

Fig.10. Chromatogram : Oxidative degradation of anagrelide

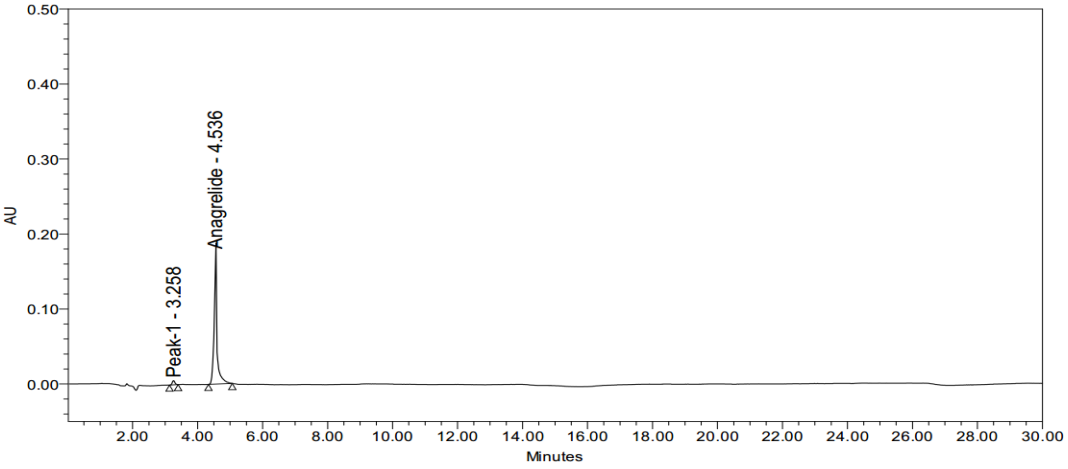

Fig.11. Chromatogram : Thermal degradation of anagrelide

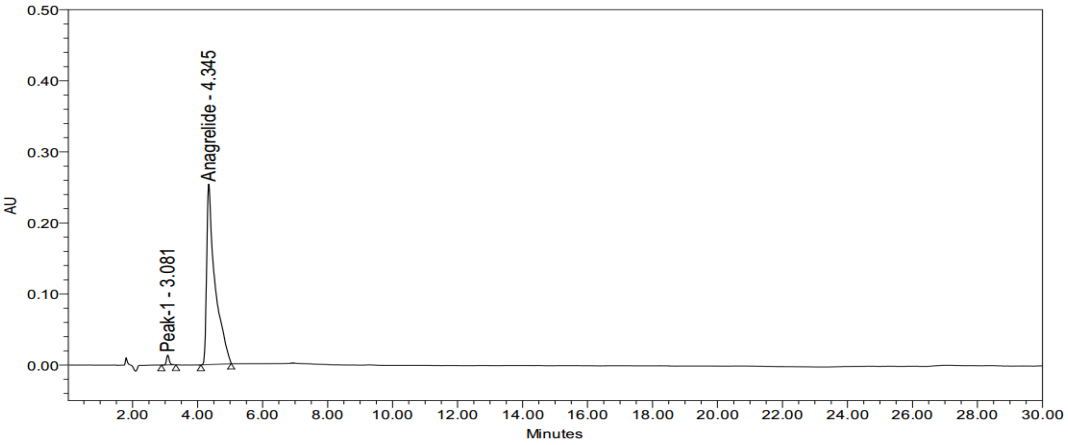

Fig.12. Chromatogram: Photolytic degradation of anagrelide

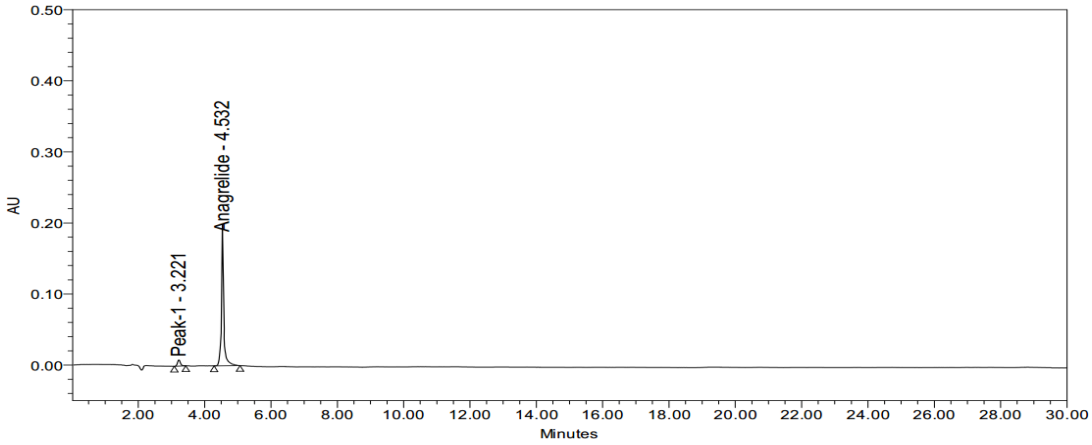

Fig.13. Chromatogram: Neutral degradation of anagrelide

Table 8. Forced degradation data for anagrelide

S.No.	Degradation	Retention time	Area	USP plate count	USP tailing	Purity angle	Purity threshold
1.	Acidic	4.538	1121328	6947	1.5	0.204	1.282
2.	Basic	4.634	1135058	6895	1.4	0.088	0.289
3.	Thermal	4.536	1174331	6249	1.5	0.181	0.369
4.	Oxidative	4.449	1163107	6455	1.4	1.133	2.582
5.	Photolytic	4.345	1216169	3426	1.5	0.128	0.585
6.	Neutral	4.532	1216729	6243	1.6	0.228	0.467

7.	Standard drug	4.595	1245618	6370	1.52	------	------

Table 9 Percentage of degradation of anagrelide

S.No.	Degradation Studies	% Degradation
1.	Acidic	9.4
2.	Basic	8.3
3.	Thermal	5.1
4.	Oxidative	6.0
5.	Photolytic	1.7
6.	Neutral	1.6

Table 10 A comparison chart of the proposed (current) method with the reported methods for determination of anagrelide

Method	Column	Mobile phase	Flow rate	Retention times	Linearity range	Run time
Proposed method	Kromasil C18 (150 x 4.6 mm; 5□m)	Phosphate buffer (pH 2.5) and acetonitrile (75:25 v/v)	**1.0 mL/min**	**4.818 min.**	50–150 µg/mL	**8 min**
Kalaichelvi *et al* [4]	Agilent Eclipse XDB C18 column (150 x 4.6 mm ; 5µ)	phosphate buffer (ph 4.0) and acetonitrile (70:30 v/v)	1.0 mL/min	5.8 min.	50–150 µg/mL	above 9 min
Venugopal *et al* [5]	Chromosil C18 column(250 mm x 4.6 mm; 5µ).	methanol, acetonitrile and water in the ratio 80:15:05(v/v)	1.0 mL/min	4.46 min.	20-120 µg/ml.	10 min
Sudhakar *et al* [6]	C18 Inertsil (250 x 4.6 mm ; 5µ)	phosphate buffer methanol: acetonitrile (90:5:5, v/v/v)	1.0 mL/min	8.376 min	0.05–152 µg /ml.	above 10 min

Ramanjaneyulu *et al* [7]	XTerra symmetry C18 (150×4.6 mm; 5µm)	acetonitrile and water (ph 3.0) in a ratio of 40:60 v/v	1.2 mL/min	2.349 min.	5-30 µg/mL	above 10 min
Kakadiya *et al* 8	Kromasil C18 (250mm x 4.6 mm; 5µm) column	methanol: phosphate buffer (ph-3) in a ratio of 70:30 v/v	1.0 mL/min	9.46 min	10-50 µg/mL	above 10 min

5. DISCUSSION ON THE RESULTS AND CONCLUSION

The aim of the present study was to develop a precise, accurate and sensitive HPLC method for the analysis of anagrelide in bulk drug and in pharmaceutical dosage forms. To analyse the component peaks, different combinations of mixtures of acetonitrile with phosphate buffer were tested as mobile phase on a Kromasil C18 (150 x 4.6 mm; 5µm) stationary phase. A binary mixture of acetonitrile and phosphate buffer (P^H 2.5) in a proportion of 25:75 v/v was proved to be the most suitable of all combinations since the chromatographic peaks were better defined and resolved and almost free from tailing. The retention time obtained for anagrelide was 4.818 min. Each of the samples was injected six times and the same retention times were observed in all cases. The peak areas of anagrelide were reproducible as indicated by low coefficient of variation. A good linear relationship (r=0.9991) was observed between the concentration of anagrelide and the respective peak areas. The regression curve was constructed by linear regression fitting and its mathematical expression was Y = 12421X - 9135 (where y gives peak area and x is the concentration of the drug). The regression characteristics are given in Table 2.3**.** When anagrelide solution containing 100 µ g /mL was analysed by the proposed method for finding out intra and inter- day variations, low coefficient of variation was observed. High recovery values obtained from the different dosage forms by the proposed method indicates the method is accurate. The absence of additional peaks indicates non- interference of common excipients used in the capsules.

The drug content in capsules was quantified using the proposed analytical method. The capsules were found to contain an average of 99.6 % of the labeled amount of the drug. The low coefficient of variation indicates the reproducibility of the assay of anagrelide in dosage forms.

The deliberate changes in the method have not much affected the peak tailing, theoretical plates and the percent assay. This indicates that the present method is robust. The lowest values of LOD and LOQ as obtained by the proposed method indicate the method is sensitive. The standard solution of the drug was stable up to 24 hours as the difference in percent assay is within limit.

6. CONCLUSION

System suitability parameters were studied with six replicates standard solution of the drug and the calculated parameters are within the acceptance criteria. The tailing factor, the number theoretical plates and HETP are in the acceptable limits.

Table 2.12 compares the present proposed method with the methods published earlier for anagrelide. An observation of the chart reveals some advantages of the proposed method. A Kromasil C18 column was used for the study. The retention time obtained is less than many other methods. The upper limit of quantitation is 150 µg/mL which is higher than three of the methods mentioned. The run time is also very less.

Hence the author concludes that the proposed HPLC method is sensitive and reproducible for the analysis of anagrelide in pharmaceutical dosage forms with short analysis time.

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