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# A new RP-HPLC method development for the estimation of an anti-thrombocythemic agent anagrelide in pure and pharmaceutical formulations

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**ABSTRACT**

*A new RP-HPLC method was developed and validated for the determination of an anti-thrombocythemic agent anagrelide in pure and pharmaceutical formulations. The separation was achieved on Peak HPLC instrument equipped with a LC 20AT pump and variable wavelength programmable UV-visible detector SPD-10AVP and a Chromosil C18 column (250 mm x 4.6 mm, 5µ) column. Methanol, acetonitrile and water in the ratio 80:15:05(v/v/v) was used as mobile phase at a flow rate of 1.0 ml/min and detection of the component was carried out at a wavelength of 260nm. 20µl of the standard or sample has been injected into the column through Hamilton syringe and the chromatogram was recorded. The retention time of anagrelide was found to be 4.46min. The plot was linear in the range of concentration 20-120µg/ml. The drug was subjected to forced degradation under different conditions such as UV light, Sun light, acidic, basic, thermal and oxidation. The drug was found to be stable in aqueous, basic and thermal conditions where as it was decomposed to some extent in the presence of acidic, peroxide, UV light and Sun light. The developed method was found to be simple, precise, accurate, rugged and robust and hence it can be used as an alternative method in assay of the anagrelide in any pharmaceutical industries.*

**Keywords:** RP-HPLC**,** Anagrelide, Linearity, LOD, LOQ and Degradation

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## INTRODUCTION

Anagrelide was a drug used for the treatment of essential thrombocytosis or overproduction of blood platelets. It also has been used in the treatment of chronic myeloid leukemia [1]. It works by inhibiting the maturation of platelets from megakaryocytes [2]. The exact mechanism of action was unclear, although it was known to be a phosphodiesterase inhibitor [3]. It was a potent ($IC_{50}$ = 36nM) inhibitor of phosphodiesterase-II. It inhibits PDE-3 and phospholipase A2 [4]. The chemical name of the drug was 6,7-dichloro-1,5-dihydroimidazo[2,1-b]quinazolin-2(3H)-one mono hydrochloride monohydrate, with molecular formula and molecular weight $C_{10}H_7Cl_2N_3O{\bullet}HCl{\bullet}H_2O$ and 310.55 g/mol respectively. The pharmaceutical preparation, agrylin 0.5 mg capsules are used for oral administration, each capsule contain 0.5 mg of anagrelide base as anagrelide hydrochloride. The inactive ingredients that are present in the formulation are Anhydrous Lactose NF, Crospovidone NF, Lactose Monohydrate NF, Magnesium stearate NF, Microcrystalline cellulose NF, Povidone USP.The molecular structure of anagrelide mono hydrochloride was given in Fig.1. An extensive literature surrey was carried out and found that there were few analytical methods such as and LC-MS [5-6], HPLC [7-8] and GC-MS [9] available for determination anagrelide in plasma.

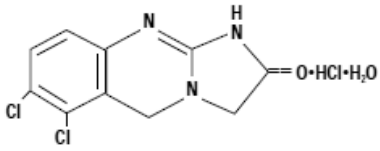


**Fig.1 Chemical structure of anagrelide hydrochloride**

## MATERIALS AND METHODS

### Instrumentation

HPLC instrument equipped with a LC 20AT pump and variable wavelength programmable UV-visible detector SPD-10AVP, 20µL Hamilton syringe and a Chromosil C18 column (250 mm x 4.6 mm, 5µ) column was used for the present investigation. A Denwar balance and Loba ultrasonic bath sonicator used for were also used for weighing the materials and degassing of the mobile phase respectively. ElicoSL 159 UV-Visible spectrophotometer was used for spectral studies. The data was analyzed by using peak software.

### Chemicals and reagents

A reference sample of anagrelide was obtained from Lupin, Mumbai. The formulations were procured from the local market. The solvents such as acetonitrile, methanol and water used for the present investigation were of HPLC grade and purchased from Merck Specialties Private Limited, Mumbai, India. A mixture of acetonitrile, methanol and water in the ratio80:15:05 (v/v/v) was used as mobile phase and used in the preparation of analytical solutions.

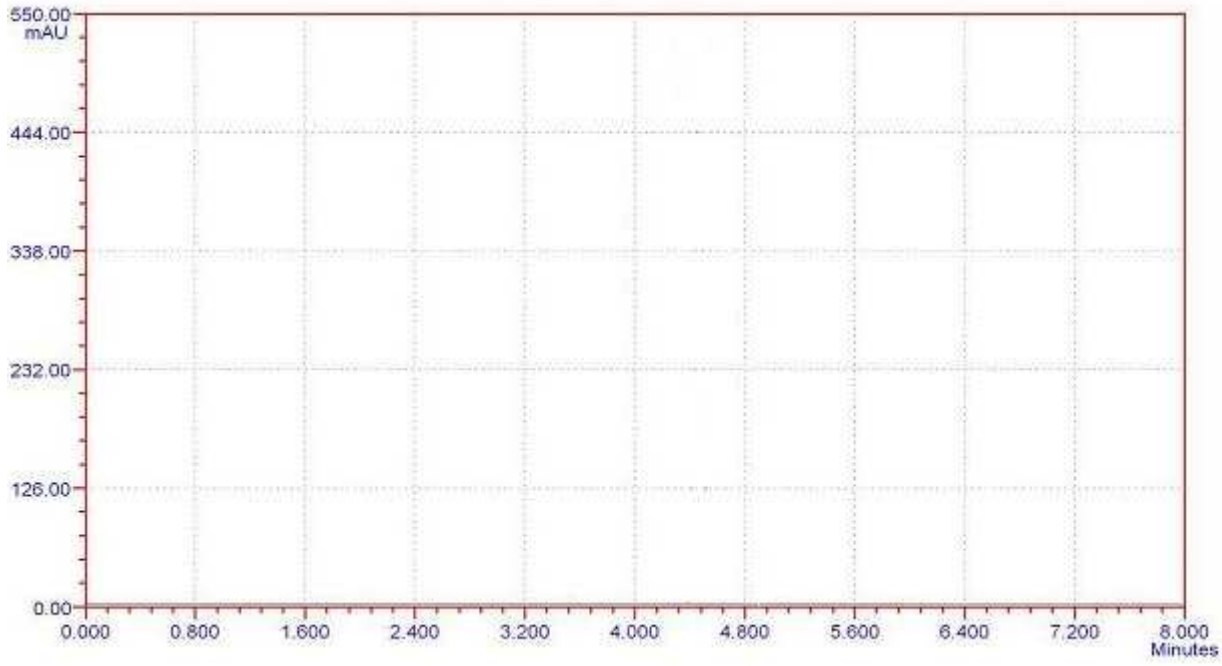


**Fig. 2 A typical chromatogram of blank solution (with out anagrelide)**

### Preparation of standard and sample solutions

For the present investigation 1000 µg/ml stock solution of anagrelide was prepared by dissolving 10mg of drug in to 10 ml of methanol and required working standard dilute solutions of anagrelide were prepared from the stock solution by proper dilution. Ten capsules of anagrelide were finely powdered; the amount of the powder was weighed equivalent to 10 mg of the drug, dissolved in 10ml of mobile phase, sonicated and filtered through a 0.45mm membrane filter. Then working sample solutions of different concentrations are prepared from the stock solution (1000µg/ml).

### Development of chromatographic method

The UV absorption spectrum of working standard solution of (80µg/ml) the anagrelide was recorded separately on UV-Visible spectrophotometer and found the maximum absorbance wavelength at 260nm. The mobile phase was allowed to pass through the column at a flow rate of 1.0 ml/min at ambient temperature for 30 min. to equilibrate the column, the response of the detector was recorded continuously at maximum wavelength 260nm and found that the base line was parallel to time axis which indicates the absence of additional peaks in the mobile phase. Different

trails were performed by injecting different volumes working standard of different concentrations at different wavelengths using different composition of mobile phase to get a reasonable retention time of the drug and valid system suitability parameters such as number of theoretical plates, tailing factor and resolution. Finally, the chromatogram of 20µl of 80µg/ml working standard at 260nm using a mobile phase of composition of 80:15:05 (v/v/v) of acetonitrile, methanol and water for a run time of 8.0min was found to be suitable for the assay analysis. Typical chromatograms of blank and standard are presented in Fig.2 and Fig.3 respectively.

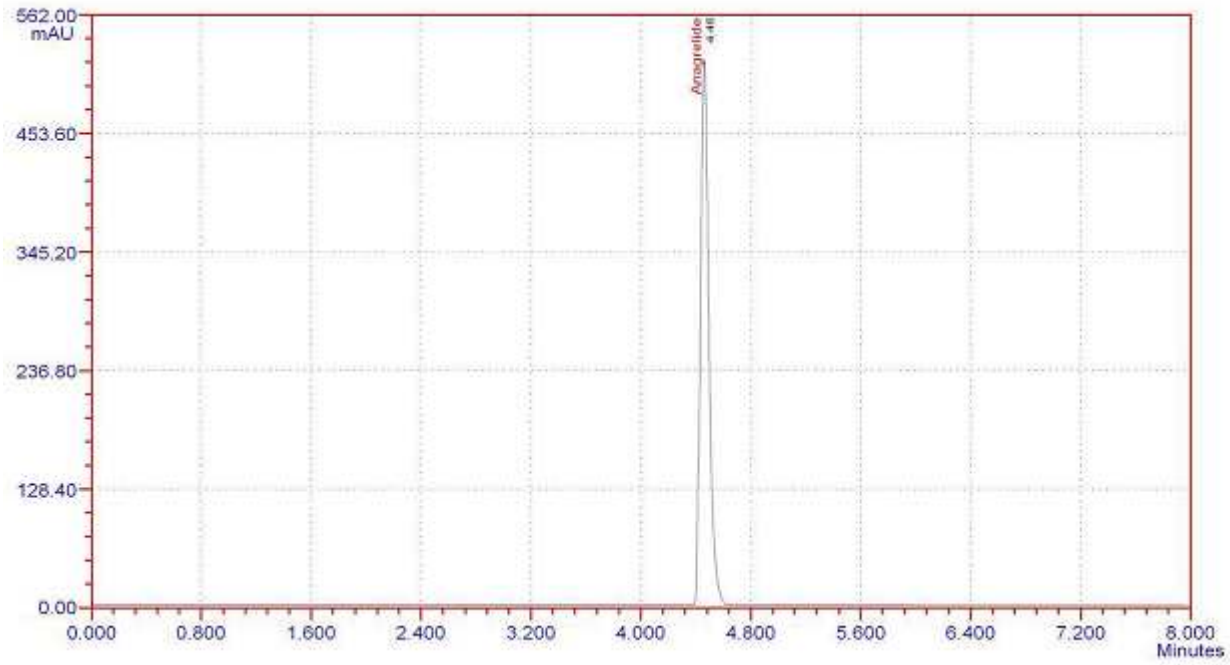


**Fig. 3 A typical chromatogram of anagrelide standard (80µg/ml)**

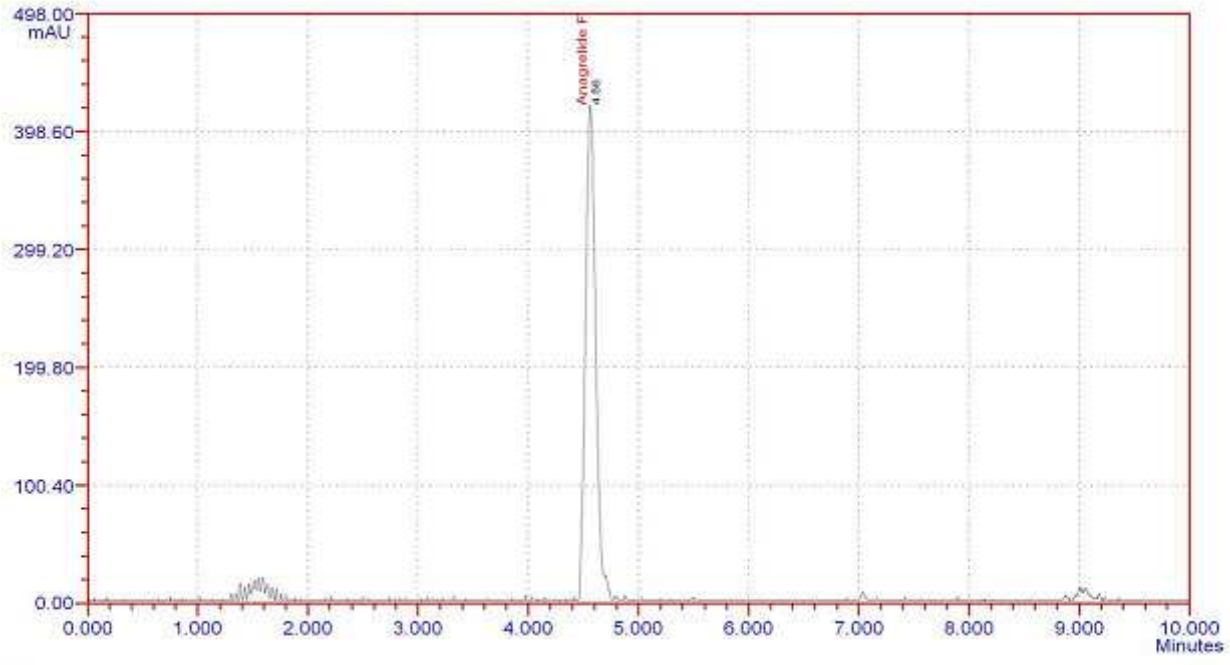


**Fig. 4 A typical chromatogram of anagrelide formulation (80µg/ml)**

**Method validation**

**System suitability**

System suitability was studied in terms of tailing factor, number of theoretical plates and resolution under each validation parameters by injecting six replicates of the standard solution (80ppm). Tailing factor and number of

theoretical plates are found to be 1.32 and 9340 respectively. The resolution between any two adjacent peaks should be more than 2.0 and it was not applicable for the present investigation.

**Specificity**

The specificity of the proposed method was studied by comparing the chromatograms of blank (Fig.2), working standard and sample solutions of the drug. It was found that there were no additional peaks in the sample chromatogram (Fig.4), hence there was no interference due to the presence of excipients in the formulation and also found good correlation between retention time and peak area of standard and sample.

**Linearity**

Linearity between average peak area of two replicates and concentration was performed by preparing six different concentrations of the standard solution of anagrelide including working concentration mentioned in experimental condition. 20µl of each concentration was injected in duplicate into the HPLC system, the corresponding chromatograms were recorded at 260nm and mean peak areas were calculated. A plot was drawn between mean peak area and concentration and it was found to be linear (Fig.5). The slope, intercept and correlation coefficient of the six measurements were calculated by least square regression method. Linearity results were presented in Table-1.

**Table-1 Linearity studies of the proposed method**

| Sample ID | Concentration (µg/ml) | Mean peak area |
|---|---|---|
| 1 | 20 | 103747 |
| 2 | 40 | 181594 |
| 3 | 60 | 270701 |
| 4 | 80 | 346683 |
| 5 | 100 | 442662 |
| 6 | 120 | 526286 |
| | Slope | 4324.6 |
| | Intercept | 7905.7 |
| | Correlation coefficient | 0.9994 |

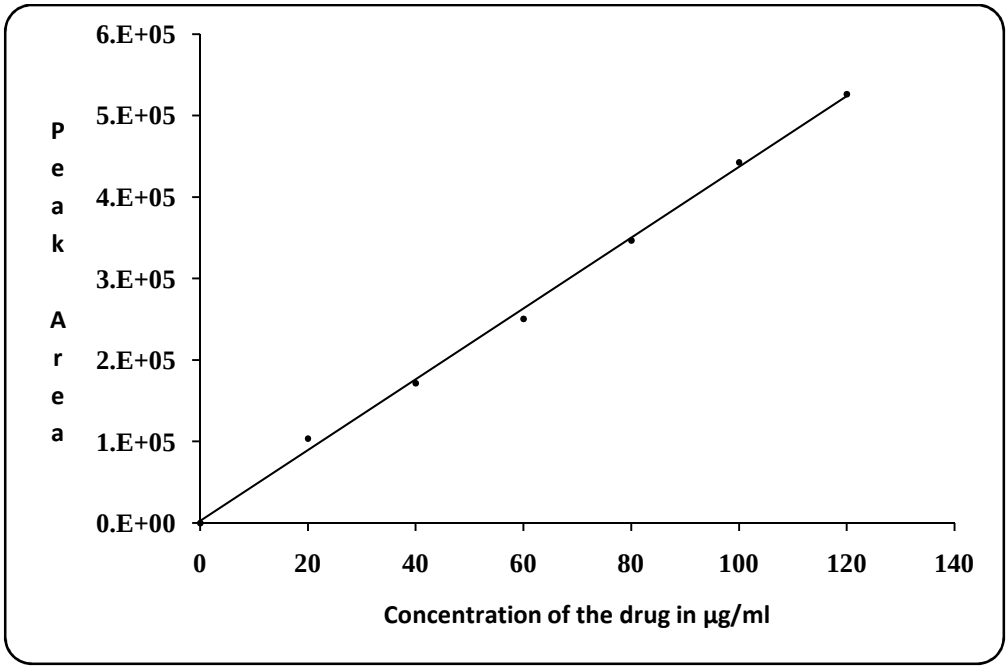


**Fig.5 A linearity plot of peak area against concentration of anagrelide in µg/ml.**

**Limit of detection and Limit of quantification**

To determine the limit of detection, the sample was dissolved in mobile phase and injected into the system until peak was diapered. After 0.25 µg/ml dilution the peak was not clearly observed. So it confirms that 0.25 µg/ml was the limit of detection. The limit of quantification was evaluated by determining the least concentration of the drug that can be quantified and found to be 0.825µg/ml.

**Precision**

Precision was the degree of repeatability of an analytical method under normal operational conditions. Precision of the method was performed as intraday precision and inter day precision. To study the intraday precision, six replicate standard solutions (80µg/ml) of anagrelide were injected and the chromatograms were obtained. The percent relative standard deviation (% RSD) of the peak area of six peaks was calculated and found to be 0.74. To study the interday precision, six replicate standard solutions (80µg/ml) of anagrelide was injected on third day of sample preparation. The percent relative standard deviation (% RSD) was found to be 0.59. The results were found to be within the acceptable criteria and were shown in Table-2.

**Table-2 Precision of the proposed method**

| Sample | Concentration | Intraday precision | | Inter day precision | |
|---|---|---|---|---|---|
| | | Sample ID | Peak area | Sample ID | Peak area |
| Anagrelide | 80µg/ml | 1 | 346683 | 1 | 347846 |
| | | 2 | 348996 | 2 | 346786 |
| | | 3 | 347509 | 3 | 342782 |
| | | 4 | 342455 | 4 | 345226 |
| | | 5 | 348563 | 5 | 347509 |
| | | 6 | 349055 | 6 | 348300 |
| Statistical Parameters | | Mean | 347276.55 | | 346408.13 |
| | | SD | 2569.33 | | 2076.08 |
| | | %RSD | 0.740 | | 0.599 |

**Accuracy**

The accuracy of the method was determined by standard addition method. A known amount of standard drug was added to the fixed amount of pre-analyzed tablet solution at three different concentration levels (50%, 100% and 150% level of the working standard) in triplicate at each level. Percent recoveries were calculated by comparing the area before and after the addition of the standard drug and results are presented in Table-3. Satisfactory recoveries ranging from 98.9 to 101.46 were obtained by the proposed method. This indicates that the proposed method was accurate.

**Table-3: Study of accuracy at three spiked levels**

| Spike level | Amount of anagrelide | | |
|---|---|---|---|
| | Taken µg/ml | Obtained µg/ml | % Recovery |
| 50% | 40 | 39.86 | 99.65 |
| | 40 | 39.56 | 98.9 |
| | 40 | 40.22 | 100.55 |
| | | | Average=99.70 |
| 100% | 80 | 79.89 | 99.86 |
| | 40 | 81.17 | 101.46 |
| | 40 | 79.51 | 99.39 |
| | | | Average=100.23 |
| 150% | 120 | 119.42 | 99.52 |
| | 120 | 121.24 | 101.03 |
| | 120 | 120.85 | 100.71 |
| | | | Average=100.42 |

*Mean average: 100.11*

**Table 4: Study of Robustness of the developed method**

| Variable | Variation | RT* | Peak area | TF* | NTP* |
|---|---|---|---|---|---|
| Standard | Not applicable | 4.46 | 346682.8 | 1.32 | 1251 |
| Flow Rate | 1.1 ml/min. | 4.46 | 347985.5 | 0.57 | 9875 |
| | 0.9 ml/min. | 4.46 | 354768.5 | 0.57 | 9547 |
| Wavelength | 265 nm | 4.46 | 342162.4 | 0.59 | 10228 |
| | 255 nm | 4.46 | 347914.7 | 0.58 | 9893 |
| Buffer pH | 4.1 | 4.46 | 344460.3 | 0.59 | 10092 |
| | 4.2 | 4.46 | 347162.7 | 0.58 | 9936 |
| %Mobile Phase Methanol: Acetonitrile : Water | 75:20:05 (v/v/v) | 4.46 | 356379.3 | 0.56 | 9429 |
| | 85:10:05 (v/v/v) | 4.46 | 343169.4 | 0.59 | 10168 |

**RT: Retention time, TF*: Tailing factor, NTP*: Number of theoretical plates, Acceptance Criteria USP Tailing Factor not more than 2.0, Theoretical Plates not less than 2000*

**Robustness**

The study of robustness was performed by slight modification in chromatographic conditions such as flow rate of the mobile phase, pH of the buffer and composition of the mobile phase. The working standard solution of anagrelide was

analyzed under these new experimental conditions. Only one parameter was changed while the others were kept unaltered. The system suitability parameters were evaluated as per the test method in all the cases and found to be within limits shown in Table-4.

**Ruggedness**

Ruggedness was the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions. Ruggedness of the proposed method was performed by injecting six replicate injections of sample solutions of anagralide and the percent of assay was determined under different columns, different systems, and different labs over a period of one week. The results were presented in Table-5.

**Table-5: Study of ruggedness of the proposed method**

| Anagralide Sample 80µg/ml | | | | | | |
|---|---|---|---|---|---|---|
| | Column-1 | Column-2 | System-1 | System-2 | Lab-1 | Lab-2 |
| Sample ID | Peak area | Peak area | Peak area | Peak area | Peak area | Peak area |
| 1 | 99.52 | 100.64 | 101.45 | 99.84 | 99.41 | 99.47 |
| 2 | 99.14 | 99.54 | 99.25 | 99.91 | 101.45 | 101.45 |
| 3 | 101.21 | 99.27 | 98.94 | 100.45 | 99.73 | 100.65 |
| 4 | 99.25 | 100.34 | 99.75 | 101.84 | 99.57 | 98.94 |
| 5 | 99.54 | 100.56 | 99.64 | 99.42 | 101.47 | 99.54 |
| 6 | 100.57 | 99.74 | 100.84 | 99.67 | 99.37 | 99.71 |
| Mean | 99.87 | 100.02 | 99.98 | 100.19 | 100.17 | 99.96 |
| SD | 0.83 | 0.57 | 0.97 | 0.88 | 1.01 | 0.92 |
| %RSD | 0.830 | 0.574 | 0.968 | 0.876 | 1.008 | 0.919 |

**Stability test**

To perform the stability of the test and the standard solution, a solution of concentration 80 µg/ml was stored at ambient temperature (± $5^0$C) for two days. On the third day the stored solution and freshly prepared solution were analyzed and the percent of assay was calculated. It was noticed that assay of these results were within the specified limits. The results of stability test were shown in Table-6.

**Table-6: Stability studies of the drug with respect to time**

| S. No | Concentration | Time (hrs) | Area | %of Assay |
|---|---|---|---|---|
| 1 | 80µg/ml | Fresh | 347509 | Not applicable |
| 2 | | 6 | 346252 | 99.64 |
| 3 | | 12 | 345224 | 99.34 |
| 4 | | 24 | 343151 | 98.75 |
| 5 | | 36 | 342488 | 98.55 |
| 6 | | 48 | 331251 | 95.32 |
| | | Average | 341673.20 | 98.32 |
| | | SD | 6021.70 | 1.73 |
| | | %RSD | 1.762 | 1.763 |

**Analysis of pharmaceutical formulations**

Pharmaceutical formulations are analyzed by the developed and validated method. Agrylin of 0.5mg/capsule and 1.0mg/capsule are chosen for the assay analysis. A sample solution of 80.0µg/ml was prepared and used for the assay analysis. The assay of the drug present in the each capsule was calculated by comparing the area of the peak of test with the standard. The results are presented in Table-7

**Table-7 Assay analysis of pharmaceutical formulations**

| Brand Name | Dosage | Amount Found | % Assay |
|---|---|---|---|
| Agrylin(Shine) | 1.0mg/capsule | 0.9848 mg/capsule | 98.48** |
| Agrylin(Shine) | 0.5mg/capsule | 0.5007 mg/capsule | 100.14** |

***Average of six determinations*

**Study of forced degradation of anagrelide**

The sample was subjected to forced degradation under different laboratory conditions such as Lab light, UV light, Thermal, acidic, basic, and peroxide then the amount of the drug that was stable under these conditions was calculated. The drug was kept in open petri dish and exposed to Lab light, UV light and kept in oven at 40°C, after 48 hours solutions were injected into the system, chromatograms were recorded and the percent of degradation of the anagralide was evaluated under the same chromatographic conditions. To determine the percent of degradation of the sample in acidic, basic and peroxide medium 300 mg of sample was dissolved in 20 ml of 0.1 N HCl or 0.1N NaOH or 3% hydrogen peroxide. After 48 hours 5 ml of the above sample solution was taken into two 25 ml

volumetric flask and separately neutralized and make up to the mark with diluent. The amount of drug that could be degraded was evaluated by comparing the peak areas of chromatograms of hydrolyzed compound with unhydrolyzed compounds.

**Table 8: Results of degradation**

| Degradation parameter | Number of additional peaks observed | Percent of peak area |
|---|---|---|
| Standard | Not applicable | Not applicable |
| 3% Peroxide | 3 | 69.4 |
| 0.1 N Basic | 3 | 83.82 |
| 0.1 N Acidic | 5 | 70 |
| Heat (40°c) | 2 | 82.02 |
| Aqueous (HPLC) | 2 | 92.8 |
| UV Light | poly | 59.07 |
| Sun light | 3 | 65.05 |

## RESULTS AND DISCUSSION

To estimate the amount of anagrelide in pure and pharmaceutical formulations by reverse phase high performance liquid chromatography under optimized chromatographic conditions the chromatograms are recorded for standard and test solutions (Fig.3 & Fig.4). The system suitability parameters are evaluated and found within the limits. A plot was drawn between concentration of the component and the instrument response and it was found to be linear (Fig.5) in the concentration range 20µg/ml to 120µg/ml with good correlation coefficient(r=0.9994). Precision and accuracy of the developed method are expressed in %RSD and % of recovery of the active pharmaceutical ingredient respectively. Low %RSD value 0.740 and 0.599 and high percent of recovery 98.90-101.46 indicates that the method was highly precise and accurate

A study was conducted between two different systems, two columns and two labs to compare the results. The system suitability parameters evaluated as per the test method are found to be within limits. The average % assay and relative standard deviation are within the limits. The change in system suitability parameters are evaluated by studying the effect of change in composition of the mobile phase, flow rate, pH of the buffer solution, wavelength and column temperature and found to be acceptable. Pharmaceutical formulations of 1.0mg and 0.5mg are analyzed by the proposed method by standard addition method. The amount of drug recovered was estimated and found to be satisfactory. High percent of mean recovery and low %RSD values indicate that the method should be successfully applied for the analysis of formulations. The drug was subjected to forced degradation under different laboratory conditions such as Lab light; UV light, Thermal, acidic, basic, and peroxide then the amount of the drug that was stable under these conditions was calculated and found satisfactory results.

## CONCLUSION

The proposed RP-HPLC method for the estimation of anagrelide in dosage forms was found to be more sensitive and highly precise. The percent of recovery of the drug (accuracy) indicates that the proposed method was selective. Hence, the developed RP–HPLC method may be used as an alternative method for routine analysis of the raw materials and formulations.

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