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METHOD DEVELOPMENT AND VALIDATION OF RP-HPLC FOR ASSAY OF TRAMETINIB IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

A simple, selective, precise and accurate High Performance Liquid Chromatographic method for the analysis of Trametinib was developed in Pharmaceutical Dosage form. Trametinib showed maximum absorption at 245nm. This method achieved by using isocratic elution with ratio ammonium acetate buffer: methanol: acetonitrile: tetrahyrofuron mobile phase at 40:30:20:10V/V. The developed HPLC method was validated for Linearity, Accuracy, Interday Precision, Specificity & Selectivity, Robustness, Solution stability, limit of detection & limit of quantification. Stability Indicating Methods were developed for Trametinib in Pharmaceutical Dosage form under hydrolytic stress condition (5N HCl, 5N NaOH); Oxidation condition (5% H_2O_2) and dry heat condition, Thermal condition, UV light. From this study, it was found that drug is not susceptible for degradation to hydrolytic condition, oxidation condition, dry heat, UV light, and thermal condition. All the developed methods were successfully applied to determine the drug in Pharmaceutical preparation.

KEYWORDS: HPLC, Trametinib, Spectrophotometric method, Pharmaceutical Dosage.

INTRODUCTION

Trametinibis chemically N-(3-{3-Cyclopropyl-5-[(2-fluoro-4-iodophenyl)amino]-6,8-dimethyl-2,4,7-trioxo-3,4,6,7-tetrahydropyrido[4,3-d] pyrimidine-1(2H)-yl}phenyl)acetamide. Trametinib (trade name Mekinist) is a cancer drug. It is a MEK inhibitor drug with anti-cancer activity.[1] It inhibits MEK1 and MEK2.[1] Trametinib had good results for metastatic melanoma carrying the BRAF V600E mutation in a phase III clinical trial. In this mutation, the amino acid valine (V) at position 600 within the BRAF protein has become replaced by glutamic acid (E) making the mutant BRAF protein constitutively active.[2] In May 2013, Trametinib was approved as a single-agent by the Food and Drug Administration for the treatment of patients with V600E mutated metastatic melanoma.[3] Clinical trial data demonstrated that resistance to single-agent Trametinib often occurs within 6 to 7 months.[4] To overcome this, Trametinib was combined with the BRAF inhibitor dabrafenib.[4] As a result of this research, on January 8, 2014, the FDA approved the combination of dabrafenib and Trametinib for the treatment of patients with BRAF V600E/K-mutant metastatic melanoma.[5]

Trametinib (Mekinist) is an inhibitor of MAPK kinase (MEK). Both drugs are registered for the treatment of patients with BRAF V600 mutation positive unrespectable or metastatic melanoma. The use of BRAF inhibitors, such as vemurafenib has been associated with prolonged survival and progression-free survival in phase 3 trials.[6,8] Trametinib resulted in improved progression-free survival and overall survival.[9] In spite of these advances, 50% of patients who are treated with BRAF inhibitors or MEK inhibitors develop resistance leading to disease progression within 6–7 months after initiation of the treatment. Since Trametinib mediates blockade of MEK, which is downstream of BRAF in the MAPK pathway, the combination of Trametinib was investigated. Combination therapy of Trametinib was associated with a longer median progression-free survival than dabrafenib monotherapy.[10] To further investigate the pharmacokinetics of both drugs and to support therapeutic drug monitoring (TDM) an assay for the simultaneous quantification of Trametinib is needed. Two bioanalytical assays for Trametinib were reported before by Mittapalli et al.[11] but without bioanalytical details and by Sparidans et al.[12] No bioanalytical assays have been described for Trametinib as far as we know. Herein we describe the first bioanalytical assay for the quantification of Trametinib that was fully validated according to the latest FDA and EMA guidelines on bioanalytical method validation.[13,14]

However, in the knowledge authors, no account has been reported for stability indicating assay method for determination of Trametinib. According to current good manufacturing practices, all drugs must be tested with a stability-indicating assay method before release. Till date, no stability-indicating HPLC assay method for the

determination of Trametinib is available in the literature. It was felt necessary to develop a stability indicating liquid chromatography (LC) method for the determination of Trametinib as bulk drug and pharmaceutical dosage form and separate the drugs from the degradation products under the International Conference on Harmonization (ICH) suggested conditions (hydrolysis, photolysis and thermal stress).[15,16] Therefore, the aim of the present study was to develop and validate a stability indicating HPLC assay method for Trametinib as bulk drug and in Pharmaceutical Dosage form as per ICH guidelines.

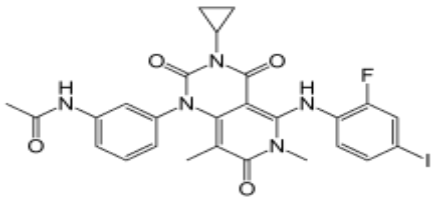

Fig 1: Structure of Trametinib

MATERIALS AND METHODS

Instrumentation and Chromatographic Conditions

Shimadzu (LC 20 AT VP) (Prominence-shimadzu scientific instruments) (for specificity and forced degradation studies) with Spin chrome (LC solutions) software was used for the analysis. The column used was Zodiac C18, 250×4.6mm ID, 5µm Particle size. Different mobile phases were tested in order to find the best conditions for the separation of Trametinib and degradents. The optimum composition of mobile phase was determined to be Phosphate Buffer: Methanol (40:60). The flow rate was set to 1 mL min-1,UV detection was carried out at 257 nm and 20µl injection volume were maintained. The mobile phase and samples were filtered using 0.45µm membrane filters. Mobile phase was degassed by ultrasonic vibrations prior to use. All determinations were performed at ambient temperature (25° C).

Preparation of standard stock solution of Trametinib for absorption maxima

10mg of Trametinib was weighed in to 100ml volumetric flask and dissolved in Methanol and then dilute up to the mark with methanol and prepare 10 µg /ml of solution by diluting 1ml to 10ml with methanol.

Preparation of mixed standard stock solution

Weigh accurately 100 mg of Trametinib in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 100 µg/ml of Trametinib was prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Assay

Preparation of Standard sample solution

Standard stock solution of Trametinib (microgram/ml) was prepared by 100 mg of Trametinib dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5min and dilute to 100 ml with mobile phase. Further dilutions are prepared in 5 replicates of 100 µg/ml of Trametinib was made by adding 1 ml of stock solution to 10 ml of mobile phase.

Preparation of Tablet sample solution

20 tablets (each tablet contains 150 mg of Trametinib) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solution (Trametinib) were prepared by dissolving weight equivalent to 100 mg of Trametinib and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 100ml with mobile phase. Further dilutions are prepared in 5 replicates of 100 µg/ml of Trametinib was made by adding 1 ml of stock solution to 10 ml of mobile phase.

Calculation

The amount of Trametinib present in the formulation by using the formula given below, and results shown in table 9.

$$\%\ \text{Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{AW}{LC} \times 100$$

Where,
AS: Average peak area due to standard preparation
AT: Peak area due to assay preparation
WS: Weight of Trametinib in mg
WT: Weight of sample in assay preparation
DT: Dilution of assay preparation.

Selection of detection Wavelength

The wavelength of maximum absorption (λ_{max}) of the drug, 10 µg/ml solution of the drug in methanol was scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against methanol as blank. The resulting spectra are shown in the figure 2 and the absorption curve shows characteristic absorption maxima at 245 nm for Trametinib.

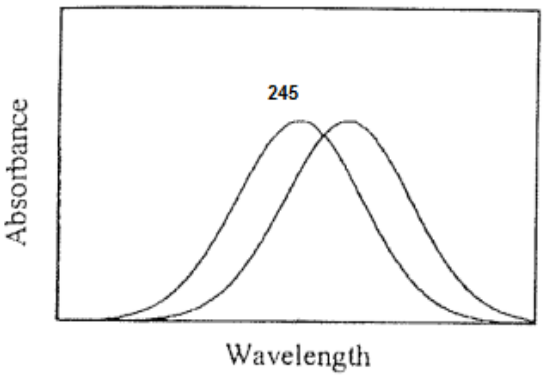

Fig. 2: Spectrum of trametinib.

Method Validation

Linearity

Linearity test solutions for the assay method were prepared from Trametinib stock solutions at five concentration levels from 60% to 140% of assay analyte concentration (60%, 80%, 100%, 120% and 140%). The peak area verses concentration data was treated by least squares linear regression analysis.

Linearity test solutions for the related substance method were prepared by dilution of stock solution to the required concentrations. The solutions were prepared at five concentration levels from LOQ to 140% of specification level (60%, 80%, 100%, 120% and 140%). Above tests were carried out for 3 consecutive days in the same concentration range for assay method. The % RSD value for the Slope and Y-intercept of the calibration curve was calculated.

Precision

The precision of the assay method was evaluated by carrying out six independent assays of Trametinib test samples against a qualified reference standard and calculate the % R.S.D of assay. The precision of the related substances method was checked by injecting six individual preparations of Trametinib (0.1 mg mL-1) % RSD was calculated. The intermediate precision of the method was also evaluated using different analyst and different instrument in the same laboratory.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its degradation impurities. The specificity of the developed HPLC method for Trametinib was carried out. Stress studies were performed for Trametinib bulk drug to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress conditions of UV light (254nm), acid (0.5N HCl, base (0.5N NaOH), Oxidation (3.0% H2O2) and heat (80°C) to evaluate the ability of the proposed method for determination of assay of Trametinib. Assay studies were carried out of stress samples against qualified Trametinib reference standard.

Accuracy

The accuracy of the assay method was evaluated in triplicate at three concentration levels 80%, 100% and 120% of test concentration (0.1 mg mL-1). The percentage of recoveries was calculated from the Slope and Y- intercept of the calibration curve obtained in the linearity study.

Ruggedness: Analyst variation and instrument variation were observed by taking 15µg/ml and their %RSD was calculated.

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered. The flow rate of the mobile phase was 1.0mL min-1.To study the effect of flow rate on the resolution, flow was changed by 0.2 units from 0.8 to 1.2 mL min-1. The effect of the column temperature on resolution was studied at 20 and 30°C instead of 25°C. The effect of wave length variation was studied at 255 and 259 instead of 257 max wave length and no variation was observed.

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

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitavely determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices and is used particularly for the determination of Impurities and/or degradation products. The limit of detection (LOD) and limit of quantitation (LOQ) were separately determined at a signal to noise ratio (S/N) of 3 and 10.

Solution stability and Mobile phase stability

The solution stability of Trametinib in the assay method was carried out by leaving both thesolutions of sample and reference standard in tightly capped volumetric flasks at roomtemperature for 48 hours. The same sample solutions were assayed for 6 hours interval up to thestudy period. The mobile phase stability was also carried out by assaying the freshly preparedsample solution against freshly prepared reference standard solution for 6 hours interval up to 48hours. Mobile phase prepared was kept constant during the study period. The % R.S.D for the assay of Trametinib was calculated during mobile phase and solution stability experiment.

RESULTS AND DISCUSSION

Optimization of Chromatographic conditions

The main objective of chromatographic method is to obtained Trametinib assay method using different mobile phases and stationary phases such as: Ammonium acetate: Methanol (ACN: THF(40:30:20:10) PH 5, Buffer(KH2PO4):THF:Methanol (55:1:44), 0.7%v/v H_3PO_4: ACETONIRILE (50:50), Phosphate buffer:MeOH (60:40) pH 5.5 & Phosphate buffer:MeOH (40:60) and stationary phases Inertsil ODS 3V(250x4.6mm) 5µm, Symmetry C18, 75mm X 4.6 mm I.D 5µ particles, & Zodiac C18 250mm X 4.6mm and ID with (250×4.6× 5µ) were used. Finally the chromatographic separation was achieved on an Zodiac C18 250mm X 4.6mm and ID with (250×4.6× 5µ) column using mixture of Phosphate buffer:MeOH (40:60v/v) as a mobile phase. The flow rate of the mobile phase was 1.0 mL min-1, at 25°C column temperature, the peak shape of the Trametinib was found to be symmetrical. In optimized chromatographic conditions of Trametinib, with tailing factor less than 2 and theoretical plates more than 2000, typical retention times

were about 2.4 minuts. Developed HPLC method was found to specific for Trametinib.

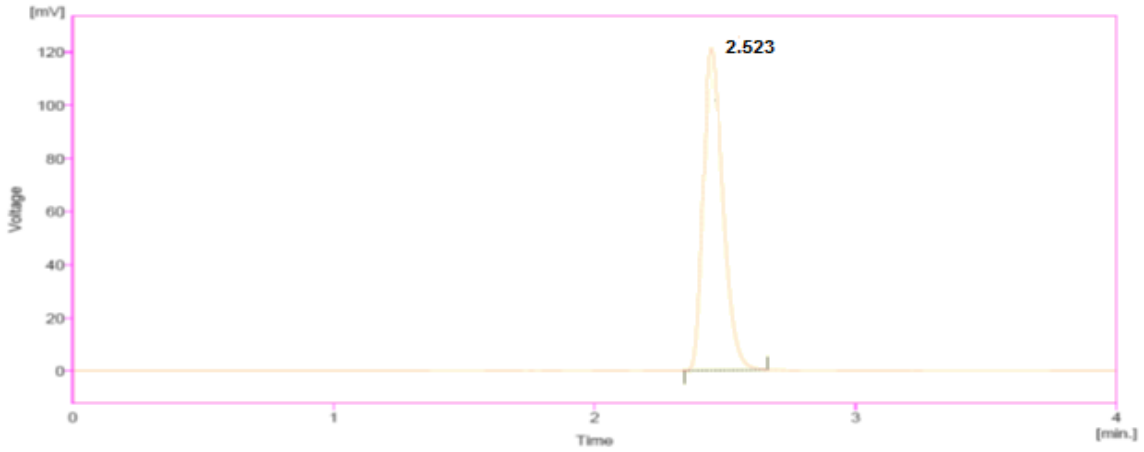

Fig. 3: Chromatogram for Specificity of Trametinib standard.

Linearity and range

Preparation of standard stock solution

Standard stock solutions of Trametinib (µg/ml) were prepared by dissolving 100 mg of Trametinib in 10 ml of mobile phase. After that filtered the solution using 0.45-micron syringe filter and sonicated for 5 min. and dilute 100ml with mobile phase and further dilutions were given in the table No.1 and Fig 4.

Acceptance criteria

The relationship between the concentration of Trametinib and area of Trametinib should be linear in the specified range and the correlation should not be less than 0.99.

OBSERVATION

The correlation coefficient for linear curve obtained between concentrations vs. Area for standard preparations of Trametinib is 0.996. The relationship between the concentration of Trametinib and area of Trametinib is linear in the range examined since all points lie in a straight line and the correlation coefficient is well within limits.

Table 1: Linearity of Trametinib.

S.No.	Conc.(µg/ml)	Area
1	60	390.656
2	80	505.508
3	100	650.922
4	120	800.647
5	140	902.268

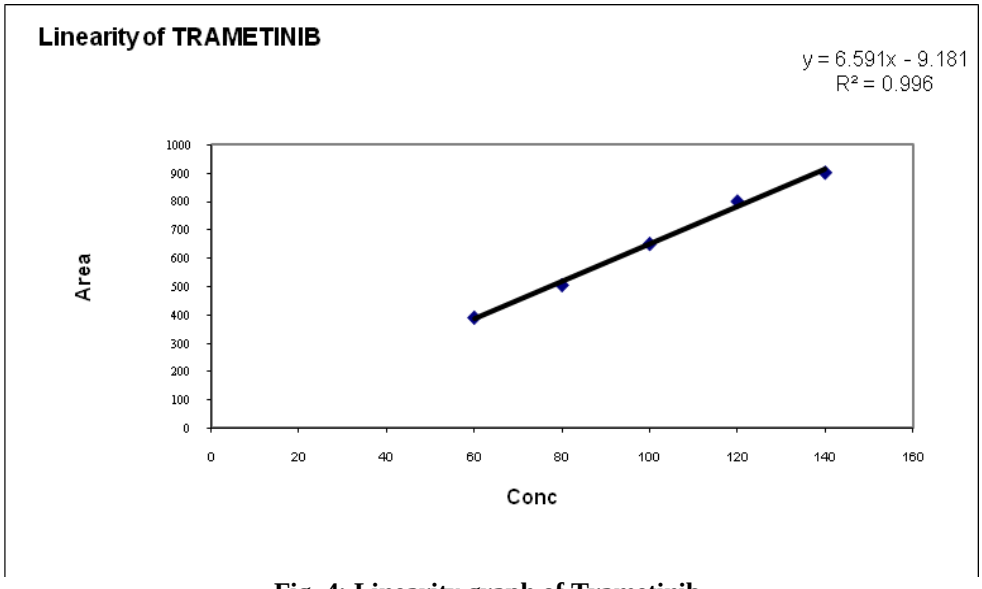

Fig. 4: Linearity graph of Trametinib.

Accuracy

Accuracy of the method was determined by Recovery studies. To the formulation (pre analysed sample), the reference standards of the drug were added at the level of 80%, 100%, 120%. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for drug is shown in table 2. To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-analyzed sample solution at three different levels 80%, 100%, 120%.

Table 2: Recovery results for Trametinib.

Recovery level	Accuracy Trametinib					Average % Recovery
	Amount taken(mcg/ml)	Area	Average area	Amount recovered (mcg/ml)	%Recovery	
80%	100 mcg/ml	439.813 434.816 441.982	438.008	99.14	99.14	99.54%
100%	120 mcg/ml	587.277 586.212 587.277	586.389	120.89	99.86	
120%	140 mcg/ml	614.51 619.688 622.308	618.676	137.71	98.37	

The % recovery of Trametinib should be between 98% and 102% and the percentages mean recovery of Trametinib is 99.54%.

Precision

Method precision

Prepared sample preparation of Trametinib as per test method and injected 6 times in to the column. The % Relative standard deviation of Assay preparations should be not more than 2.0%.

Test results for Trametinib showing that the %RSD of Assay results are within limits. The results were shown in table3.

Table 3: Results for Method precision of Trametinib.

Injection	Retention time (min)	Peak area	Theoretical plates (TP)	Tailing factor (TF)
1	2.47	647.79	4428	1.610
2	2.52	647.23	4439	1.671
3	2.52	645.963 2.46 | 647.79	4439	1.611
4	2.48	645.534	4415	1.610
5	2.52	641.381	4417	1.614
6	2.52	640.922	4379	1.612
Mean	2.50	644.80	-	-
SD	0.0077	2.948	-	-
%RSD	0.31	0.46	-	-

Limit of Detection

$$LOD = \frac{3.3\sigma}{S}$$

Where, σ = the standard deviation of the response
S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

The LOD for this method was found to be 15.83 µg/ml & area 104.57 for Trametinib.

Limit of Quantification

$$LOQ = \frac{10\sigma}{S}$$

Where,
σ = the standard deviation of the response
S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

The LOQ for this method was found to be 109.65 µg/ml & area 316.81 for Trametinib.

Robustness

Chromatographic conditions variation

To demonstrate the robustness of the method, prepared solution as per test method andinjected at different variable conditions like using different conditions like flow rate and wavelength. System suitability parameters were compared with that of method precision.

Acceptance criteria
The system suitability should pass as per the test method at variable conditions.

Table 4: Results of Trametinib for Robustness (0.8 ml/min).

NAME	Robustness	Rt	AREA	Th.Plates	ASSYMETRY
TRAMETINIB	Flow Rate(0.8ml/min)	2.182	698.387	4743	1.632
	Flow Rate(1.2ml/min)	2.74	599.569	4479	0.647
	Wavelength (255nm)	2.641	675.111	4296	0.527
	Wavelength (259nm)	2.153	521.286	4287	0.367

From the observation it was found that the system suitability parameters were within limit at all variable conditions.

Ruggedness
The ruggedness of the method was studied by the determining the analyst to analyst variation by performing the Assay by two different analysts. The % Relative standard deviation of Assay values between two analysts should be not more than 2.0%.

Table 5: Results for Ruggedness.

TRAMETINIB	%Assay
Analyst 01	100.21
Anaylst 02	99.80
%RSD	0.289%

From the observation the %RSD between two analysts Assay values not greater than 2.0%, hence the method was rugged.

Stability Indicating Tests for Trametinib by Rp-Hplc
Specificity by forced degradation study
Forced degradation of Trametinib has been carried out, to confirm that during stability study or throughout the shelflife, any degradation product if found will not interfere in the main peak. In addition, the forced degradation study will help to identify the type of degradation pathway (whether oxidative, alkali hydrolysis, acid hydrolysis, water hydrolysis, photolytic and dry heat) for each of the degradant. Sample as such transferred in to a 100ml volumetric flask and make up the volume with diluent. Trametinib Place boas such 1ml is transferred into a 100ml volumetric flask and diluted to volume with diluent and check it for the degradation studies.

Acid Stressed sample
1ml of Trametinib sample was transferred into a 100ml volumetric flask.2ml of 0.1N HCl was added and kept in water bath for 1hrs at 80°C Cooled. Neutralized with 0.1N NaOH and diluted to volume with diluent, from this solution target concentration was prepared and injected. The total % degradation are presented in Table 6.

Table 6: Forced degradation study by Acid Stress.

NAME	Rt	AREA	Th.Plates	ASSYMETRY
TRAMETINIB	2.153	521.286	4287	0.367

Alkali Stressed sample
1ml of Trametinib sample was transferred into a 100ml volumetric flask. 2ml of 0.1N NaOH was added and kept in water bath for 1hrs at 80°C Cooled. Neutralized with 0.1N HCl and diluted to volume with diluent, from this solution target concentration was prepared and injected. The total % degradation are presented in Table 7.

Table 7: Forced degradation study by Alkali Stress.

NAME	Rt	AREA	Th.Plates	ASSYMETRY
TRAMETINIB	2.051	436.878	5255	0.455

Peroxide stressed sample
1 ml of Trametinib sample was transferred into a 100 ml volumetric flask 2 ml of hydrogen peroxide (3.0%v/v) was added, kept in water bath for 2hrs at 80°C. Cooled and diluted to volume with diluent, from this solution target concentration was prepared and injected. The total % degradation are presented in Table 8.

Table 7: Forced degradation study by Peroxide Stress.

NAME	Rt	AREA	Th.Plates	ASSYMETRY
TRAMETINIB	2.051	436.878	5255	0.455

Thermal Stressed (Dry heat) sample (in a hot air oven)

1ml of Trametinib sample was transferred into a 100ml volumetric flask and kept sample in an oven for 2hours at 80°C, cooled to room temperature, then diluted to volume with diluent, from this solution target concentration was prepared and injected. The total % degradation are presented in Table 8.

Table 8: Forced degradation study by thermal Stress.

NAME	Rt	AREA	Th.Plates	ASSYMETRY
TRAMETINIB	2.051	436.878	5255	0.455

UV light exposed sample

1ml of Trametinib sample was transferred into a 100ml volumetric flask and exposed the sample to UV light for 2 hours and then diluted to volume with diluent, from this solution target concentration was prepared and injected. The total % degradation represented in table 9.

Table 8: Forced degradation study by UV light exposed.

NAME	Rt	AREA	Th.Plates	ASSYMETRY
TRAMETINIB	2.051	436.878	5255	0.455

Table -9: Assay Results of Trametinib.

TRAMETINIB		
	Standard Area	Sample Area
Injection-1	442.878	436.878
Injection-2	441.488	436.878
Injection-3	442.967	421.286
Injection-4	439.797	436.878
Injection-5	439.655	436.878
Average Area	440.444	435.982
Average weight	250.1 mg	
Standard weight	100.1 mg	
Sample weight	166.66mg	
Label amount	150mg	
std.purity	99.8	
Amount found in mg	149.63 mg	
Assay(%purity)	99.75%	

CONCLUSION

HPLC method was developed for estimation of Trametinib in Pharmaceutical Dosage form. The developed HPLC method was validated for Linearity, Accuracy, Interday Precision, Specificity & Selectivity, Robustness, Solution stability, limit of detection & limit of quantification. Stability Indicating Methods were developed for Trametinib in Pharmaceutical Dosage form. Stability Indicating Methods were developed for Trametinib in Pharmaceutical Dosage form under hydrolytic stress condition (5N HCL, 5N NaOH); Oxidation condition (5% H2O2) and dry heat condition, Thermal condition, UV light. From this study, it was found that drug is not susceptible for degradation to hydrolytic condition, oxidation condition, dry heat, UV light and thermal condition. All the developed methods were successfully applied to determine the drugs in Pharmaceutical preparation.

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