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Development and validation of a RP-HPLC method for estimation of Dolutegravir in API form and combined oral solid dosage form

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Abstract

An efficient and simple HPLC method has been developed and validated for the determination of Trametinib in bulk and was applied on marketed Trametinib products. The mobile phase used for the chromatographic runs consisted of Acetonitrile and Phosphate buffer (0.01M, pH-3.2) in the ratio of 30:70 v/v. The separation was achieved on a Symmetry C18 ODS (4.6mm×250mm) 5µm particle size column using isocratic mode. Drug peak were well separated and were detected by a UV detector at 246 nm. The method was linear at the concentration range 6–14 µg/ml for Trametinib. The method has been validated according to ICH guidelines with respect to system suitability, specificity, precision, accuracy and robustness. Trametinib limit of detection (LOD) and limit of quantification (LOQ) were 0.487µg/ml and 1.477µg/ml respectively.

Keywords: Trametinib, RP-HPLC, Accuracy, Precision, Robustness, ICH Guidelines

Introduction

Trametinib is an orally bioavailable inhibitor of mitogen-activated protein kinase (MAP2K; MAPK/ERK kinase; MEK) 1 and 2, with potential antineoplastic activity. Upon oral administration, Trametinib [1] specifically binds to and inhibits MEK 1 and 2, resulting in an inhibition of growth factor-mediated cell signaling and cellular proliferation in various cancers. MEK 1 and 2, dual specificity serine/threonine and tyrosine kinases often upregulated in various cancer cell types, play a key role in the activation of the RAS/RAF/MEK/ERK signaling pathway that regulates cell growth. Trametinib [2] is an anticancer agent which causes apoptosis (or programmed cell death) and inhibits cell proliferation, which are both important in the treatment of malignancies. Trametinib is a reversible, allosteric inhibitor of mitogen-activated extracellular signal regulated kinase 1 (MEK1) and MEK2 activation and of_ MEK1_ and MEK2 kinase activity. MEK proteins are upstream regulators of the extracellular signal-related kinase (ERK) pathway, which promotes cellular proliferation. Trametinib [3] helps with melanoma with the BRAF V600E or V600K as the mutation results in the constitutive activation of the BRAF pathway which includes MEK1 and MEK2. Trametinib is indicated for the treatment of unresectable or metastatic melanoma with BRAF V600E or V600K mutations, as detected by an FDA-approved test [FDA]. The IUPAC Name of Trametinib is N-[3-[3-cyclopropyl-5-(2-fluoro-4-iodoanilino)-6, 8-dimethyl-2, 4, 7-trioxopyrido [4, 3-d] pyrimidin-1-yl] phenyl] acetamide. The Chemical Structure of Trametinib is as follows.

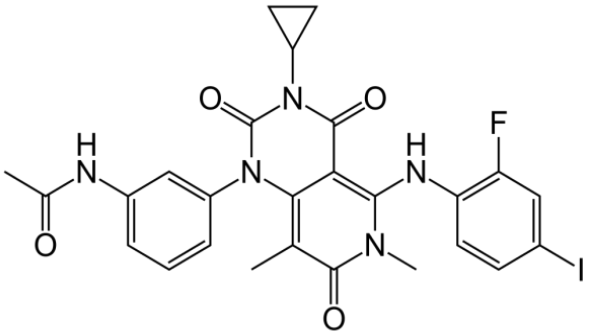

Fig 1: Chemical Structure of Trametinib

Experimental

Table 1: List of Equipments

S. No.	Instruments/Equipments/Apparatus
1.	HPLC WATERS with Empower2 Software with Isocratic with UV-Visible Detector.
2.	T60-LABINDIA UV – Vis spectrophotometer
3.	High Precision Electronic Balance
4.	Ultra Sonicator (Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry C_{18} Column, 250 mm x 4.6 mm and 5µm particle size
7.	P^H Analyser (ELICO)
8.	Vaccum Filtration Kit (Labindia)

Table 2: List of Chemicals used

S.No.	Name	Grade	Manufacturer/Supplier
1.	HPLC grade water	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	HPLC	Loba Chem; Mumbai.
3.	Ethanol	A.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	HPLC	Loba Chem; Mumbai.
5.	DMSO	A.R.	Sd fine-Chem ltd; Mumbai
6.	DMF	A.R.	Sd fine-Chem ltd; Mumbai

Method Development

HPLC Instrumentation & Conditions: The HPLC system employed was **HPLC WATERS** with Empower2 Software with Isocratic with UV-Visible Detector [4].

Standard Preparation for UV-Spectrophotometer Analysis

The Standard Stock Solutions-10 mg of Trametinib standard was transferred into 10 ml volumetric flask, dissolved & make up to volume with Methanol. Further dilutions were done by transferring 1 ml of the above solution into a 10ml volumetric flask and make up to volume with methanol to get 10ppm concentration.

It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Trametinib, so that the same wave number can be utilized in HPLC UV detector for estimating the Trametinib.

Selection of Chromatographic Methods

The proper selection depends upon the nature of the sample, (ionic or ion stable or neutral molecule) its molecular weight and stability. The drugs selected are polar, ionic and hence reversed phase chromatography [5] was selected.

Optimization of Column

The method was performed with various columns like Hypersil C_{18} column, X- bridge column and X-terra (4.6 ×150mm, 5µm particle size), Symmetry C18 ODS (4.6mm×250mm) 5µm particle size Column was found to be ideal as it gave good peak shape and resolution[6] at 1ml/min flow.

Mobile Phase Optimization

Initially the mobile phase tried was Water: Methanol and Water: Acetonitrile and Methanol with TEA Buffer with varying proportions. Finally, the mobile phase was optimized to Acetonitrile: Phosphate buffer (0.01M, pH-3.2) in the ratio of 30:70 respectively.

Preparation of Mobile Phase

Accurately measured 300 ml (300%) of HPLC Grade Acetonitrile and 700 ml of Phosphate buffer (70%) were mixed and degassed in a digital ultra sonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filter.

Preparation of 0.01M Potassium dihydrogen orthophosphate Buffer Solution

About 1.36086grams of Potassium dihydrogen orthophosphate was weighed and transferred into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC Grade water. The pH was adjusted to 3.20 with diluted orthophosphoric acid.

Diluent Preparation: Mobile phase can be used as diluent.

Assay

Preparation of the Trametinib standard solution

Accurately weigh and transfer 10 mg of Trametinib, working standard into a 10ml of clean dry volumetric flasks add about 7ml of diluent and sonicate to dissolve and removal of air completely and make volume up to the mark with the diluent. Further pipette 0.1ml of Trametinib from stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

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.

Preparation of Sample Solution

Take average weight of Tablet and crush in a mortar by using pestle and taken weight 10 mg equivalent weight of Trametinib 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.

Procedure

Further pipette 0.1ml of Trametinib from above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

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

%ASSAY =

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

Analytical Method Validation

Validation

Validation [7] is a process of establishing documented evidence which provide a high degree of assurance that specific activity will consistently produce a desired result or product meeting its predetermined specification and quality characteristics.

System Suitability

System suitability is the evaluation of the components of an analytical system to show that the performance of a system meets the standards required by a method. A system suitability [8] evaluation usually contains its own set of parameters. For chromatographic assays, these may include tailing factor, resolution, precision, capacity factor time and theoretical plates.

Accuracy

For preparation of 50% Standard stock solution

Further pipette 0.05ml of Trametinib from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

For preparation of 100% Standard stock solution

Further pipette 0.1ml of Trametinib from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

For preparation of 150% Standard stock solution

Further pipette 0.15 ml of Trametinib from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

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 Trametinib and calculate the individual recovery and mean recovery values [9].

Acceptance criteria

The %RSD [10] for each level should not be more than 2.

C. Precision

Repeatability

Preparation of Trametinib for Precision

Further pipette 0.1 ml of Trametinib from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent

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.

D. Ruggedness

To evaluate the intermediate precision[11] of the method, Precision was performed on different days by maintaining same conditions.

Procedure

DAY 1

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

DAY 2

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

The % RSD for the area of five standard injections results should be not more than 2%.

E. Linearity

Preparation of Level – I (6µg/ml of Trametinib)

Further pipette 0.06 ml of Trametinib from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of Level – II (8µg/ml of Trametinib)

Further pipette 0.08 ml of Trametinib from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of Level – III (10µg/ml of Trametinib)

Further pipette 0.1ml of Trametinib from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of Level – IV (12µg/ml of Trametinib)

Further pipette 0.12ml of Trametinib from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of Level – V (14µg/ml of Trametinib)

Further pipette 0.14ml of Trametinib from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

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[12].

Acceptance Criteria: Correlation coefficient should be not less than 0.999.

F. Limit of Detection

The detection limit [13] is determined by the analysis of samples with known concentration of analyte and by establishing that minimum level at which the analyte can reliably detected.

G. Limit of Quantitation

The quantification limit [14] is generally determined by the

analysis of sample with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

F. Robustness

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

Effect of Variation of flow Rate

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded.

Effect of Variation of mobile phase organic composition

The sample was analyzed by variation of mobile phase i.e. Acetonitrile: Phosphate Buffer was taken in the ratio and 70:30, 75:25 instead of 65:35, remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded.

Forced Degradation Studies

The specificity [15] of the method can be demonstrated by applying stress conditions using acid, alkaline, peroxide, thermal, UV, water degradations. The sample was exposed to these conditions the main peak of the drug was studied for peak purity that indicating the method effectively separated the degradation [16] products from the pure active ingredient.

Acid Degradation Studies

To 1 ml of Trametinib stock, 1 ml of 2N HCl was added and refluxed for 30 min at 60 ºC. The resultant solution was neutralized with 1 ml 2N NaOH and makeup to final volume to obtain (10µg/ml) solution. Cool the solution to room temperature and filtered with 0.45µm membrane filter. A sample of 20µl was injected into the HPLC system, and the chromatograms were recorded to assess the stability of the sample.

Alkali Degradation Studies

To 1 ml of stock solution of Trametinib 1 ml of 2N sodium hydroxide was added and refluxed for 30 min at 60 ºC. The resultant solution was neutralized with 1 ml 2N HCl and makeup to final volume to obtain (10µg/ml) solution. Cool the solution to room temperature and filtered with 0.45µm membrane filter. The sample of 20µl was injected into the system, and the chromatograms were recorded to an assessment of sample stability.

Oxidation Degradation Studies

To 1 ml of stock solution of Trametinib 1 ml of 20% hydrogen peroxide (H_2O_2) was added separately. The solution was kept for 30 min at 60ºC. For HPLC study, the resultant solution was diluted to obtain (10µg/ml) solution. Cool the solution to room temperature and filtered with 0.45µm membrane filter. A sample of 20µl solution was injected into the system, and the chromatograms were recorded to assess the stability of the sample.

Dry Heat Degradation Studies

The 1 ml of standard drug solution was placed in the oven at 60ºC for 6h to study dry heat degradation. For HPLC study, the resultant solution was makeup to final volume to obtain (10µg/ml) solution. Cool the solution to room temperature and filtered through a 0.45µm membrane filter. A sample of 20µl solution was injected into the system, and the chromatograms were recorded for the assessment of sample stability.

Photo Degradation Studies

The photo stability of the drug was studied by exposing the stock solution to UV light for 1day or 200Watt-hours/m2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain (10µg/ml) solution and filtered with 0.45µm membrane filter. A sample of 20µl solution was injected into the system, and the chromatograms were recorded for the assessment of sample stability.

Water Degradation Studies

To 1 ml of stock solution of Trametinib, 1 ml of distilled water was added. The solution was kept aside for 30 min at 60 ºC. For HPLC study, the resultant solution was diluted to obtain (10µg/ml) cool the solution to room temperature and filtered with 0.45µm membrane filter. A sample of 20µl was injected into the HPLC system, and the chromatograms were recorded for the assessment of sample stability.

Results and Discussion

Method Development

Selection of Wavelength

The detection wavelength was selected by dissolving the drug in mobile phase to get a concentration of 10µg/ml for individual and mixed standards. The resulting solution was scanned in U.V range from 200-400nm. The UV spectrum[17] of Trametinib was obtained and the Trametinib showed absorbance's maxima at 246nm. The UV spectra of drug are follows:

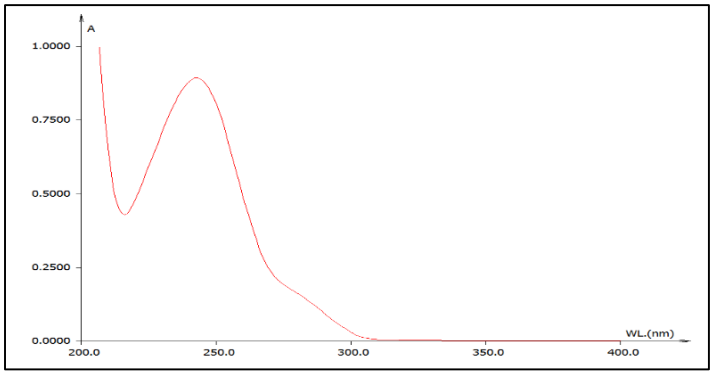

Fig 2: UV Spectrum of Trametinib (246nm)

Observation

While scanning the Trametinib solution we observed the maxima at 246nm. The UV spectrum has been recorded on T60-LAB INDIA make UV-Vis spectrophotometer model UV-2450.

Optimized Chromatographic Conditions

Mobile phase : Acetonitrile: Phosphate buffer (0.01M, pH-3.2) (30:70v/v)
Column : Symmetry C18 ODS (4.6mm×250mm) 5µm particle size
Flow rate : 1 ml/min
Wavelength : 246 nm
Column temp : Ambient
Injection Volume : 20 µl
Run time : 10 minutes

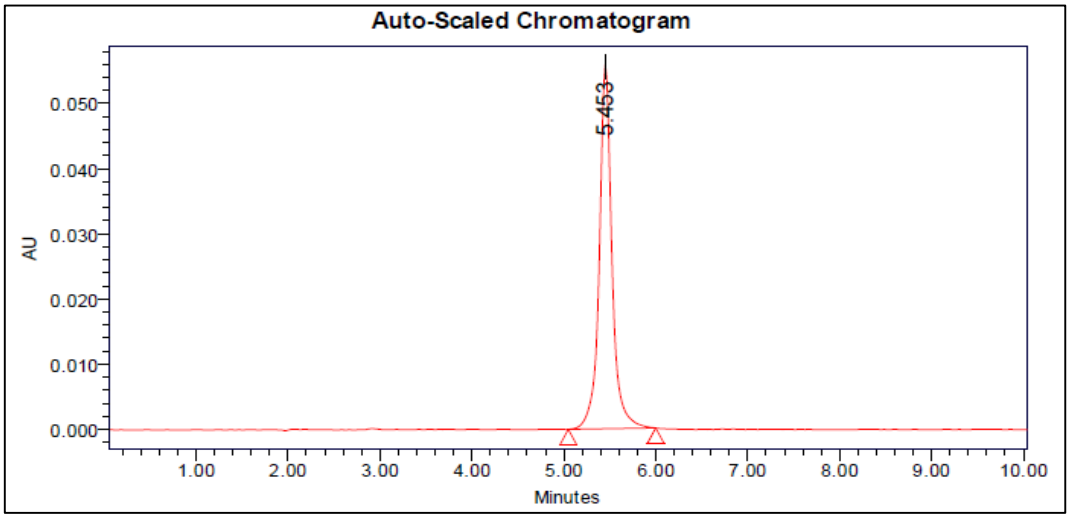

Fig 3: Optimized Chromatographic Condition

Validation of Method

System Suitability

Table 3: Observation of System Suitability Parameters

S. No.	Parameter	Trametinib
1.	Retention Time (min)	5.453
2.	Theoretical Plates	6967
3.	Tailing factor	1.12
4.	Peak Area (AUC)	647856

The system suitability parameters [18] were found to be within the specified limits for the proposed method.

Specificity

The ICH documents [19] define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components. Analytical method was tested for specificity to measure accurately quantitates Trametinib in drug product.

%ASSAY =

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

The % purity of Trametinib in present in the marketed pharmaceutical dosage form was found to be 99.85%.

Linearity

Chromatographic Data for Linearity Study

Table 4: Chromatographic Data for Linearity Study of Trametinib

Concentration µg/ml	Average Peak Area
6	468784
8	615798
10	768759
12	925748
14	1078765

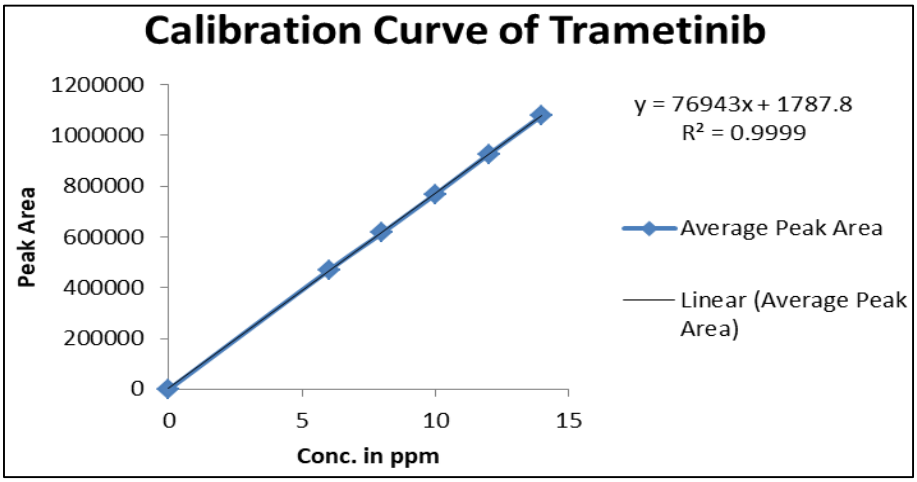

Fig 4: Calibration Curve of Trametinib

Precision

The precision [20] 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.

Repeatability

Obtained Six (6) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table 5: Results of Repeatability for Trametinib

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Trametinib	5.419	645784	83685	6825	1.05
2	Trametinib	5.405	642589	84932	6849	1.09
3	Trametinib	5.478	643658	85847	6845	1.08
4	Trametinib	5.466	648759	86295	6839	1.09
5	Trametinib	5.493	649657	86587	6895	1.07
6	Trametinib	5.466	647854	87853	6874	1.10
Mean			**646383.5**			
Std. Dev			**2853.319**			
%RSD			**0.441428**			

Intermediate Precision/Ruggedness

Table 6: Results of Intermediate precision for Trametinib (Analyst 1)

S. No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Trametinib	5.484	636854	84863	6758	1.09
2	Trametinib	5.493	637489	84759	6726	1.08
3	Trametinib	5.406	635762	84685	6749	1.09
4	Trametinib	5.419	636984	84697	6698	1.07
5	Trametinib	5.446	634856	84258	6728	1.08
6	Trametinib	5.452	639689	84753	6699	1.08
Mean			**636939**			
Std. Dev.			**1649.149**			
% RSD			**0.258918**			

Table 7: Results of Intermediate precision Analyst 2 for Trametinib

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USPP late Count	USP Tailing
1	Trametinib	5.491	628985	85698	6985	1.09
2	Trametinib	5.482	624879	85479	6899	1.07
3	Trametinib	5.416	625846	85748	6928	1.06
4	Trametinib	5.482	623568	85647	6874	1.09
5	Trametinib	5.495	628985	85246	6984	1.07
6	Trametinib	5.427	628473	85924	6872	1.08
Mean			**626789.3**			
Std. Dev.			**2340.636**			
% RSD			**0.373433**			

Accuracy

Accuracy [21] at different concentrations (50%, 100%, and 150%) was prepared and the % recovery was calculated.

Table 8: The accuracy results for Trametinib

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	386559	5	5.00	100.000%	
100%	768536	10	9.965	99.650%	100.130%
150%	1164522	15	15.111	100.740%	

The results obtained for recovery [22] at 50%, 100%, 150% are within the limits. Hence method is accurate.

Limit of Detection

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

LOD= 3.3 × σ / s

Where,
σ = Standard deviation of the response
S = Slope of the calibration curve
Result: 0.487µg/ml

Limit of Quantitation

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 response

S = Slope of the calibration curve

Result: 1.477µg/ml

Robustness

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

Table 9: Results for Robustness of Trametinib

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	648759	5.484	6845	1.08
Less Flow rate of 0.9 mL/min	635248	5.599	6786	1.09
More Flow rate of 1.1 mL/min	659865	4.576	6528	1.05
Less organic phase	625986	7.415	6689	1.03
More organic phase	615869	3.827	6354	1.01

Forced Degradation Studies

The specificity of the method can be demonstrated by applying stress conditions using acid, alkaline, peroxide, thermal, UV, water degradations [25]. The sample was exposed to these conditions the main peak of the drug was studied for peak purity that indicating the method effectively separated the degradation products from the pure active ingredient.

Table 10: Results of Forced Degradation Studies for Trametinib

S. No.	Stress Condition	Peak Area	% of Degraded Amount	% of Active Amount	Total % of Amount
1	Standard	648759	0	100%	100%
2	Acidic	539378.232	16.86	83.14	100%
3	Basic	603540.497	6.97	93.03	100%
4	Oxidative	545217.063	15.96	84.04	100%
5	Thermal	616450.801	4.98	95.02	100%
6	Photolytic	533344.773	17.79	82.21	100%
7	Water	625079.296	3.65	96.35	100%

Summary and Conclusion

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 246nm and the peak purity was excellent. Injection volume was selected to be 20µl which gave a good peak area. The column used for study was Symmetry C18 ODS (4.6mm×250mm) 5µm particle size because it was giving good peak. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time. Mobile phase is Acetonitrile: Phosphate buffer (0.01M, pH-3.2) (30:70v/v) was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study. Methanol was selected because of maximum extraction sonication time was fixed to be 10min at which all the drug particles were completely soluble and showed good recovery. Run time was selected to be 10min because analyze gave peak around 5.453min and also to reduce the total run time.

The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range. The analytical method was found linearity over the range of 6-14ppm of the Trametinib Magnesium Trihydrate target concentration. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

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