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Method Development and Validation for the Quantitative Estimation of Trametinib in API Form and Marketed Tablet Dosage Form by RP-HPLC

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Abstract

A simple and rapid reverse phase-high performance liquid chromatography (RP-HPLC) method was developed and validated for quantitative determination of Trametinib in bulk drug and marketed formulations. Trametinib was analyzed by using reverse phase Develosil ODS HG-5 RP C_{18}, 5µm, 15cm x 4.6mm i.d., with mobile phase consisting of Methanol and Phosphate buffer (0.02M, pH-3.6) in the ratio of 45:55%v/v. The flow rate was set 1.0 ml/min and the analysis was performed at wavelength 255 nm using Ultra Violet (UV) detector at ambient temperature. The method was validated according to Guidelines of ICH. The retention time for Trametinib was around 3.254 minutes. The calibration curves were linear (r 2- 0.9995) over a concentration range from 12.0 to 28.0µg/ml. Limit of detection (LOD) and Limit of quantitation (LOQ) were found to be 5.004µg/ml and 15.164µg/ml respectively. The developed method was successfully applied to estimate the amount of Trametinib in bulk form and Marketed Pharmaceutical Dosage form.

Keywords: Trametinib, ICH Guidelines, Method Development, Validation, Accuracy, Precision

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 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[3] is used alone or in combination with Dabrafenib (Tafinlar) to treat a certain type of melanoma (a type of skin cancer) that cannot be treated with surgery or that has spread to other parts of the body. The IUPAC name of Trametinib is N- [3- [3-cyclo propyl-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 shown in the following.

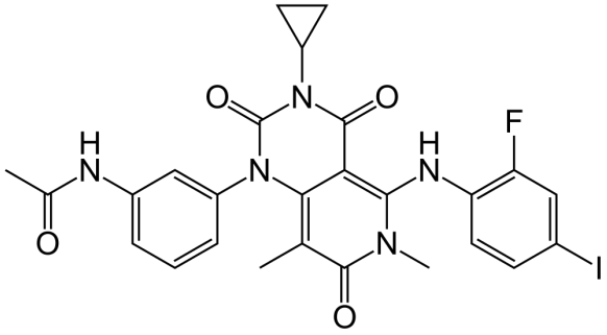

Fig1: Chemical Structure of Trametinib

Literature survey[33-36] revealed a few methods reported for determination of Trametinib in bulk drug as well as pharmaceutical preparation. In this research, a new sensitive and rapid HPLC method was developed for the determination of Trametinib in bulk and pharmaceutical dosage forms, and this method was validated according to ICH and FDA guidelines[27,32].

Experimental

Instruments Used

Table 1: List of Instrument used

S. No.	Instruments/Equipments/Apparatus
1.	HPLC with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2.	T60-LAB INDIA UV – Vis spectrophotometer
3.	Electronic Balance (SHIMADZU ATY224)
4.	Ultra Sonicator (Wensar wuc-2L)
5.	Thermal Oven
6.	Develosil ODS HG-5 RP C18, 5µm, 15cmx4.6mm i.d.
7.	P^H Analyzer (ELICO)
8.	Vacuum filtration kit (BOROSIL)

Chemicals / Reagents Used

Table 2: List of Chemicals used

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	HPLC Grade Water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
3.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
4.	Hydrochloric Acid	99.9	A.R.	Sd fine-Chem ltd; Mumbai
5.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
6.	Sodium Hydroxide	99.9	A.R.	Sd fine-Chem ltd; Mumbai
7.	Ethanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai
8.	Octanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai

Method Development

Wavelength Detection:

The detection wavelength was selected by dissolving the drug in mobile phase[4] 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 of Trametinib was obtained and the Trametinib showed absorbance's maxima at 255nm. The UV spectra of drug are follows:

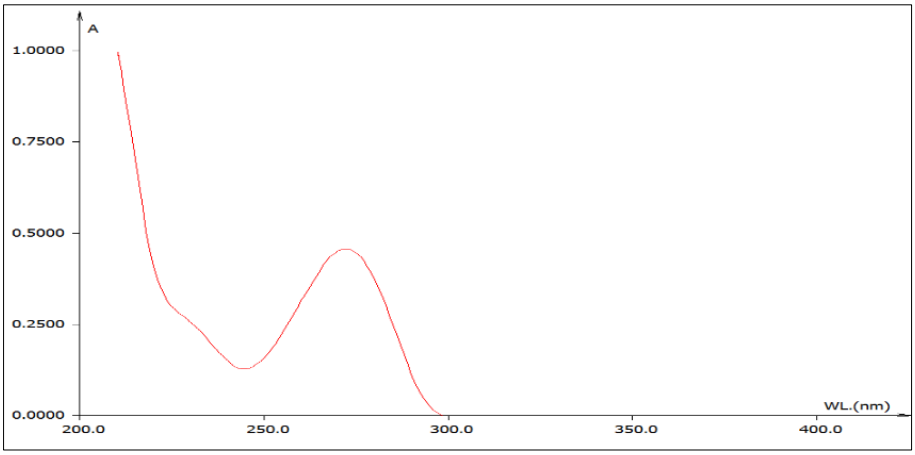

Fig 2: UV Spectrum of Trametinib

Observation: While scanning the Trametinib solution we observed the maxima at 255nm. The UV spectrum[5] has been recorded on T60-LAB INDIA make UV – Vis spectrophotometer model UV-2450.

Table 3: Trials for the Method Development and Results

S.No.	Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
1	Symmetry C_{18}, 5µm, 25cmx4.6mm i.d.	ACN : Water = 70 : 30	0.8 ml/min	255nm	Early elution of peak	Method rejected
2	Waters C_{18}, 5µm, 25cmx4.6mm i.d.	Methanol: ACN = 40 :60	1.0 ml/min	255nm	Tailing Peaks	Method rejected
3	Waters C_{18}, 5µm, 25cmx4.6mm i.d.	ACN: Phosphate buffer (0.02M) = 70:30	1.0 ml/min	255nm	Low resolution peak	Method rejected
4	Develosil ODS HG-5 RP C_{18}, 5µm,15cmx4.6mm i.d.	Methanol : Phosphate buffer (0.01M) = 50:50 (pH-3.8)	1.0 ml/ min	255nm	Many Peaks	Method rejected
5	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.	Methanol : Phosphate buffer (0.02M) = 65:35 (pH-2.6)	1.0 ml/min	255nm	Many Peaks	Method rejected
6	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.	Methanol : Phosphate buffer (0.02M) = 45:55 (pH-3.6)	1.0 ml/min	255nm	Good Peaks	Method Accepted

Optimized chromatographic method:

Table 4: Optimized Chromatographic Conditions

Mobile phase	Methanol: Phosphate buffer (0.02M, pH-3.6) = 45:55
Column	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.
Column Temperature	Ambient
Detection Wavelength	255 nm
Flow rate	1.0 ml/ min.
Run time	07 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	20µl
Type of Elution	Isocratic

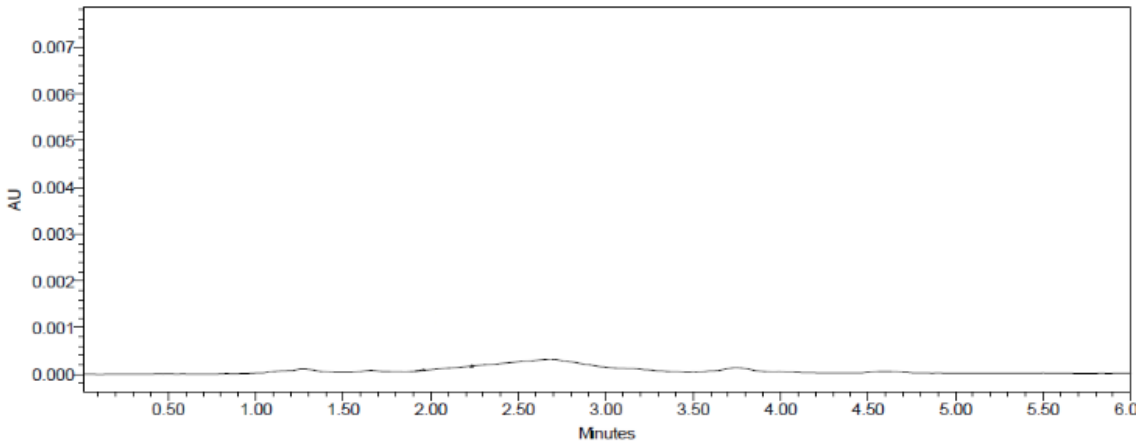

Fig 3: Chromatogram of Blank Solution

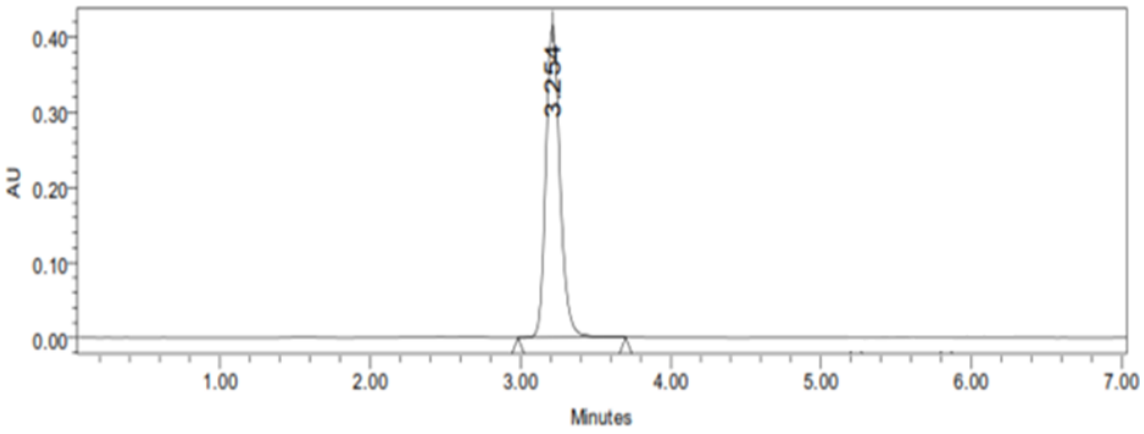

Fig 4: Chromatogram of Trametinib in Optimized Chromatographic Condition

Preparation of standard solution:
10 mg of Trametinib working standard was accurately weighed and transferred into a 10 ml clean dry volumetric flask. Add about 7 ml of diluents[6] and sonicate to dissolve it completely and volume was made up to the mark with the same solvent which gave stock solution of 1000 ppm.
Further pipette 1 ml of the above stock solution into a 10 ml volumetric flask was diluted up to the mark with diluents (100 ppm solution).
Further 1 ml of prepared 100 ppm solution was pipetted into a 10 ml volumetric flask and was diluted up to the mark with diluents which gave 10 ppm Trametinib working standard solution. The solution was mixed well and filtered through 0.45µm filter.

Preparation of sample solution:
Twenty tablets were taken and the average weight was calculated as per the method prescribed in I.P. The weighed tablets were finally powdered and triturated well. A quantity of powder of Trametinib equivalent to 10mg were transferred to clean and dry 10 ml volumetric flask and 7 ml of HPLC[7] grade methanol was added and the resulting solution was sonicated for 15 minutes. Make up the volume up to 10 ml with same solvent. Then 1 ml of the above solution was diluted to 10 ml with HPLC grade methanol. One ml (0.1 ml) of the prepared stock solution diluted to 10 ml and was filtered through membrane filter (0.45µm) and finally sonicated to degas.

Preparation of 0.02M Potassium dihydrogen orthophosphate Solution:
About 2.72172grams 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 3.60 with diluted orthophosphoric acid.

Preparation of mobile phase:
550ml of Phosphate buffer (0.02M) pH 3.60 and 450ml of HPLC Grade Methanol were mixed well and degassed in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration.

Final result & discussion: The selected and optimized mobile phase was Phosphate Buffer: Methanol = 55:45% v/v (pH-3.6) and conditions optimized were flow rate (1.0 ml/minute), wavelength (255nm), Run time was 07 mins. Here the peaks were separated and showed better resolution, theoretical plate count and symmetry. The proposed chromatographic conditions[8] were found appropriate for the quantitative determination of the Trametinib.

Analytical method validation
The optimized method for determination of Trametinib has been validated as per International Conference of Harmonisation (ICH) guidelines Q2 (R1) for evaluating system suitability, specificity, precision, accuracy, linearity, limit of detection (LOD), limit of quantitation (LOQ) and robustness.

System Suitability: System suitability testing[9-11] is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table-5 & 6.

Table 5: Data of System Suitability Test

S.No.	Injection No.	RT	Area	USP Plate Count	USP Tailing
1	Injection 1	3.253	284568	7368	1.26
2	Injection 2	3.254	285684	7295	1.25
3	Injection 3	3.215	283659	7346	1.27
4	Injection 4	3.297	284754	7394	1.29
5	Injection 5	3.253	283695	7425	1.25
6	Injection 6	3.213	284578	7385	1.27
Mean			284489.7	7368.833	1.265
S.D			752.5617		
%RSD			0.26453		

Table 6: System Suitability Results for Trametinib (Flow rate)

S.No.	Parameter	Limit	Result
1	Asymmetry	T ≤ 2	Trametinib = 0.12
2	Theoretical plate	N > 2000	Trametinib = 7258
3	Tailing Factor	(Tf) < 2	Trametinib = 1.25

Linearity: To evaluate the linearity[12], serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 0-28µg/ml for Trametinib. The prepared solutions were filtered through Whatman filter paper (No.41). From these solutions, 20µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve[13] was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

Plotting of Calibration Graphs: The resultant areas of linearity peaks are plotted against Concentration.

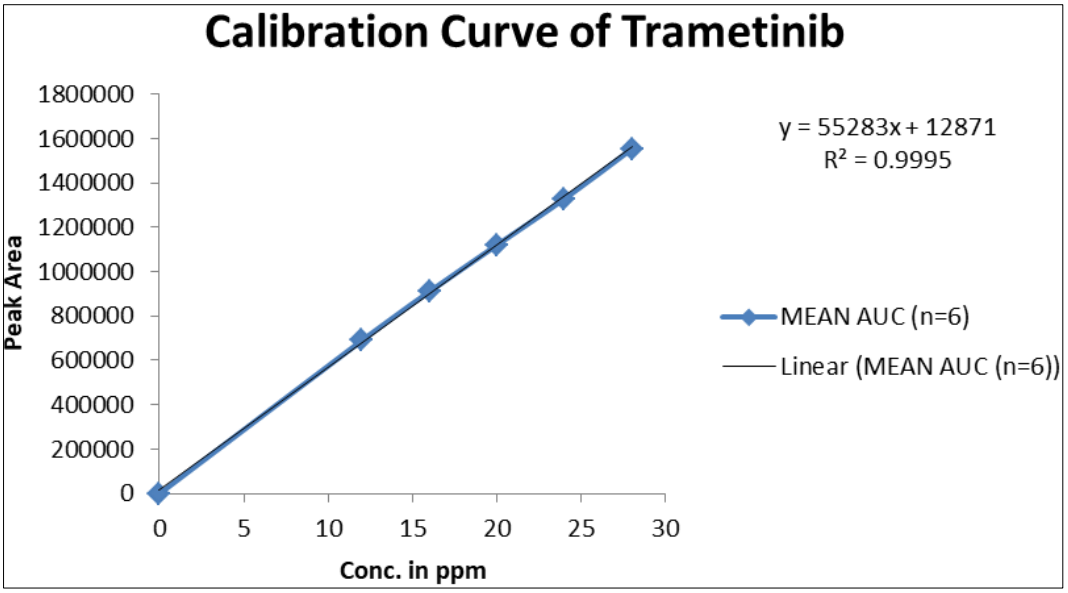

Fig 5: Standard Curve for Trametinib

Observation: Linearity range[14] was found to be 0-28µg/ml for Trametinib. The correlation coefficient was found to be 0.9995, the slope was found to be 55283 and intercept was found to be 12871 for Trametinib.

Table 7: Linearity Readings for Trametinib

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
12	690316
16	910621
20	1121057
24	1328903
28	1554666

Accuracy:

Recovery Study*:*

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Trametinib were taken and 3 replications of each has been injected to HPLC system. From that percentage recovery values[15] were calculated from the linearity equation[16-18] y = 55283x + 12871. The results were shown in table-8.

Table 8: Accuracy results of Trametinib

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	8	8.064107	458679	99.867	Mean= 100.4113% S.D. = 0.473694346 % R.S.D.= 0.471753
S_2 : 80 %	8	7.843532	446485	100.637	
S_3 : 80 %	8	8.19449	465887	100.73	
S_4 : 100 %	10	9.892661	559767	99.41	Mean= 100.6646667% S.D. = 1.166369295 R.S.D.= 1.158667
S_5 : 100 %	10	9.978655	564521	100.868	
S_6 : 100 %	10	10.19623	576549	101.716	
S_7 : 120 %	12	11.85907	668476	99.878	Mean= 100.4637% S.D. = 0.51154309 % R.S.D. = 0.509181
S_8 : 120 %	12	12.16785	685546	100.69	
S_9 : 120 %	12	12.18644	686574	100.823	

Observation: The mean recoveries were found to be 100.411, 100.664 and 100.463% for Trametinib. The limit for mean % recovery is 98-102% and as both the values are within the limit, hence it can be said that the proposed method was accurate.

Precision: The precision[19-22] of each method was ascertained separately from the peak areas obtained by actual determination of six replicates of a fixed amount of drug Trametinib. The percent relative standard deviations were calculated for Trametinib are presented in the Table-9.

a) Repeatability

Table 9: Repeatability Results of Trametinib

HPLC Injection Replicates	AUC for Trametinib
Replicate – 1	285479
Replicate – 2	284571
Replicate – 3	286954
Replicate – 4	283261
Replicate – 5	285964
Replicate – 6	284259
Average	285081.3
Standard Deviation	1318.666
% RSD	0.462558

Observation: The repeatability study which was conducted on the solution having the concentration of about 20µg/ml for Trametinib (n =6) showed a RSD of 0.462558% for Trametinib. It was concluded that the analytical technique showed good repeatability[23].

b) Intermediate Precision / Ruggedness

Table 10: Ruggedness Results for Trametinib

Conc. of Trametinib (API) (µg/ml)	Observed Conc. of Trametinib (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
8	8.21	0.76	8.23	0.46
10	10.37	0.33	10.36	0.57
12	12.56	0.23	12.56	0.75

Observation: Intraday and interday studies[24-25] show that the mean RSD (%) was found to be within acceptance limit (≤2%), so it was concluded that there was no significant difference for the assay, which was tested within day and between days. Hence, method at selected wavelength was found to be precise.

Robustness: Robustness is defined as the capacity of that method to be unaffected by even small deliberate changes that occur in the method parameters. The evaluation of robustness[26] of a method is done by varying the chromatographic parameters such as pH, temperature, flow rate, mobile phase proportions change, ionic strength etc., and determining any possible effect on the results obtained by that method.

Table 11: Result of Method Robustness Test for Trametinib

Change in Parameter	% RSD
Flow (0.8 ml/min)	0.554
Flow (1.2 ml/min)	0.867
More Organic	0.886
Less Organic	0.817
Wavelength of Detection (257 nm)	0.813
Wavelength of detection (253 nm)	0.794

Observation: Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & organic phase (±5%) studied to determine the robustness of the method are also in favour of (Table-11, % RSD < 2%) the developed RP-HPLC method for the analysis of Trametinib (API).

LOD: The limit of detection (LOD) is the lowest concentration of analyte in a sample which can be detected, but not quantitated. LOD[28] is a limit test that specifies whether an analyte is above or below a certain value. Signal-to-noise ratio of three-to-one is used to determine LOD.

L.O.D. = 3.3 (SD/S).

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Observation: The LOD was found to be 5.004µg/ml for Trametinib.

LOQ: The Limit of Quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. Signal-to-noise ratio of ten-to-one is used to determine LOQ.

L.O.Q. = 10 (SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Observation: The LOQ[29] was found to be 15.164µg/ml for Trametinib.

Assay: – Assay[30] refers to chromatography-based purity assay where a compound of unknown activity or purity is compared to a reference standard with precisely determined bioactivity or purity.

$$\text{Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Average weight} = \text{mg/tab}$$

Where:

AT = Test Preparation Peak Area

AS = Standard preparation Peak Area

WS = Working standard weight taken in mg

WT = Sample weight taken in mg

DS = Standard solution dilution

DT = Sample solution dilution

P = Working standard percentage purity

The assay was performed as explained in the previous chapter. The results[31] which are obtained are following:

Table 12: Recovery Data for Estimation Trametinib in Mekinist Tablet

Brand name of Trametinib	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Mekinist Tablets (Novartis)	2mg	1.498 (± 0.398)	99.396 (± 0.265)

Result & Discussion: The amount of drug in Mekinist Tablet was found to be 1.498 (± 0.398) mg/tab for Trametinib & % Purity was 99.396 (± 0.265) %.

Summary and conclusion

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Trametinib, different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Develosil ODS HG-5 RP C18, 5µm, 15cmx4.6mm i.d. was preferred because using this column peak shape, resolution and absorbance were good. Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Trametinib it is evident that most of the HPLC work can be accomplished in the wavelength range of 240-300 nm conveniently. Further, a flow rate of 1.0 ml/min & an injection volume of 20µl were found to be the best analysis. The result shows the developed method is yet another suitable method for assay which can help in the analysis of Trametinib in different formulations.

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Trametinib in bulk drug and pharmaceutical dosage forms. The %RSD values were within

2 and the method was found to be precise. The results expressed in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods. This method can be used for the routine determination of Trametinib in bulk drug and in Pharmaceutical dosage forms.

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