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Development and validation for the quantitative determination of tivozanib in bulk form and marketed pharmaceutical dosage form by RP-HPLC

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ABSTRACT

A simple, rapid, specific and accurate reverse phase high performance liquid chromatographic method has been developed for the validated of Tivozanib in bulk as well as in marketed pharmaceutical dosage form. This separation was performed on a Symmetry ODS (C18) RP Column, 250 mm x 4.6 mm, 5µm column with Acetonitrile, Methanol and 0.1% OPA in the ratio of 60:30:10 as mobile phase at a flow rate of 1.0 mL min−1 with UV detection at 235 nm; the constant column temperature was Ambient. The run time under these chromatographic conditions was less than 6.0 min. The retention time of Tivozanib was found to be 2.570min. The calibration plot was linear over the concentration range of 6–14 µg mL−1 with limits of detection and quantification values of 0.8 and 0.24ng mL−1 respectively. The mean % assay of marketed formulation was found to be 99.79%, and % recovery was observed in the range of 98-102%. Relative standard deviation for the precision study was found <2%.The developed method is simple, precise, specific, accurate and rapid, making it suitable for estimation of Tivozanib in bulk and marketed pharmaceutical dosage form dosage form.

Keywords: Tivozanib, RP-HPLC, Validation, Accuracy, Precision, Robustness, ICH Guidelines.

INTRODUCTION

Tivozanib is an orally bioavailable inhibitor of vascular endothelial growth factor receptors (VEGFRs) 1, 2 and 3 with potential antiangiogenic and antineoplastic activities. Tivozanib [1] binds to and inhibits VEGFRs 1, 2 and 3, which may result in the inhibition of endothelial cell migration and proliferation, inhibition of tumor angiogenesis and tumor cell death. VEGFR tyrosine kinases, frequently overexpressed by a variety of tumor cell types, play a key role in angiogenesis. Tivozanib is a Kinase Inhibitor. The mechanism of action of Tivozanib is as a Tyrosine Kinase Inhibitor. Renal cell carcinoma (RCC) is responsible for 3% of cancer cases and is one of the 10 most common cancers in adults. The average age of diagnosis is between ages 65 to 74. Tivozanib, also known as FOTIVDA, is a kinase inhibitor developed to treat adult patients with relapsed or refractory advanced renal cell carcinoma (RCC) after prior failed systemic therapies. It was approved on March 10, 2021 by the FDA. Marketed by Aveo Oncology, Tivozanib [2] is a promising therapy for individuals with RCC who have not been treated successfully with other therapies. Tivozanib inhibits growth factor receptors, treating renal cell carcinoma. In mice and rats, Tivozanib inhibits tumour angiogenesis, tumour growth, and vascular permeability. Tivozanib was shown to frequently cause

hypertension in clinical trials; hypertension must be managed before initiating therapy. Cardiac QT segment prolongation was reported in a Tivozanib cardiac safety study, however the reactions were not considered clinically serious.8 In clinical studies, levels of serum soluble VEGFR2 (sVEGFR2) decreased with time and this effect increased with Tivozanib [3] exposure, and sVEGFR2 may serve as a pharmacodynamic marker of VEGFR inhibition. The IUPAC Name of Tivozanib is 1-[2-chloro-4-(6, 7-dimethoxy quinolin-4-yl) oxy phenyl]-3-(5-methyl-1, 2-oxazol-3-yl) urea. The Chemical Structure of Tivozanib is shown in fig-1.

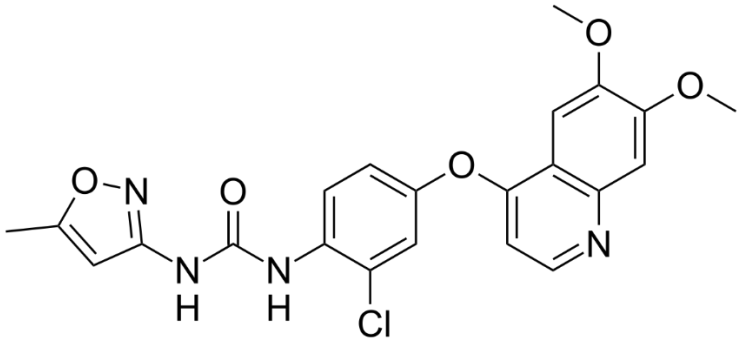

Fig 1: Chemical Structure of Tivozanib

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.	Symmetry ODS RP C_{18},5µm, 15mm x 4.6mm 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.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
3.	Dipotassium hydrogen orthophosphate	96%	A.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
5.	Potassium dihydrogen orthophosphate	99.9%	A.R.	Sd fine-Chem ltd; Mumbai
6.	Sodium hydroxide	99.9%	A.R.	Sd fine-Chem ltd; Mumbai
7.	Hydrochloric acid	99.9%	A.R.	Loba Chem; Mumbai.
8.	Hydrogen Peroxide	99.9%	A.R.	Loba Chem; Mumbai.

METHOD DEVELOPMENT

Selection of Wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase [4] diluting with the same solvent.(After optimization of all conditions) for UV analysis. It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Tivozanib, so that the same

wave number can be utilized in HPLC UV detector for estimating the Tivozanib. The scanned UV spectrum [5].

Sample & Standard Preparation for the UV-Spectrophotometer Analysis

25 mg of Tivozanib standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase.

Further dilution was done by transferring 0.5 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

Preparation of Mobile Phase

600ml of HPLC Grade Acetonitrile, 300ml of HPLC Grade Methanol and 100ml 0.1% OPA were mixed well and degassed in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration.

Optimization of Chromatographic Conditions

The chromatographic conditions [6] were optimized by different means. (Using different column, different mobile phase, different flow rate, different detection wavelength & different diluents for sample preparation etc.

Table 3: Summary of Process Optimization

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Methanol : Acetonitrile = 40 : 60	1.0ml/min	235nm	Very Low response	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Methanol : Acetonitrile = 55 : 45	1.0ml/min	235nm	Low response	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Acetonitrile : Water = 50:50	1.0ml/min	235nm	Tailing peaks	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Methanol : Water = 70:30	1.0ml/min	235nm	Resolution was not good	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	ACN : Methanol: 0.1% OPA = 70:25:5	1.0ml/min	235nm	Tailing peak	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	ACN : Methanol: 0.1% OPA = 60:30:10	1.0ml/min	235nm	Nice peak	Method accepted

Method Validation

Validation of an analytical procedure [7-10] is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications. Method validation provides an assurance of reliability during normal use, and is sometime referred to as "the process for providing documented evidence that the method does what it is intended to do."

Accuracy

The accuracy [11] of an analytical method is the closeness of the test results obtained by that method to the true value. This is sometimes termed trueness. It is recommended that accuracy should be determined using a minimum of nine determinations over a minimum of the three concentration levels, covering the specified range (3 concentrations/3 replicates each of total analytical procedures).

Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is repeated to multiple samplings of a homogeneous sample. The precision [12] of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements.

i) Repeatability

ii) Intermediate Precision

iii) Reproducibility

Specificity

Specificity [13] is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix such as impurities, degradation products and matrix components. It must be demonstrated that the analytical method is unaffected by the presence of spiked materials (impurities and/or excipients).

Linearity

Linearity is the ability of the method to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to analyte concentration within a given range [14]. It should be established initially by visual examination of a plot of signals as a function of analyte concentration of content. If there appears to be a linear relationship, test results should be established by appropriate statistical methods. Data from the regression line provide mathematical estimates of the degree of linearity [15]. The correlation coefficient, y-intercept, and the slope of the regression line should be submitted.

Range

The range of an analytical procedure is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the procedure as

written. The range is normally expressed in the same units as test results (e.g., percent) obtained by the analytical procedure [16].

Detection Limit and Quantitation Limit

The Detection Limit [17] is defined as the lowest concentration of an analyte in a sample that can be detected, not quantified. The Quantitation Limit is 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 analytical procedures.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal usage. Robustness [18] may be determined during development of the analytical procedure.

System Suitability Testing

System suitability testing [19-23] 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. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. They are especially important in the case of chromatographic procedures.

RESULTS AND DISCUSSION

Method Development

Selection of Wavelength

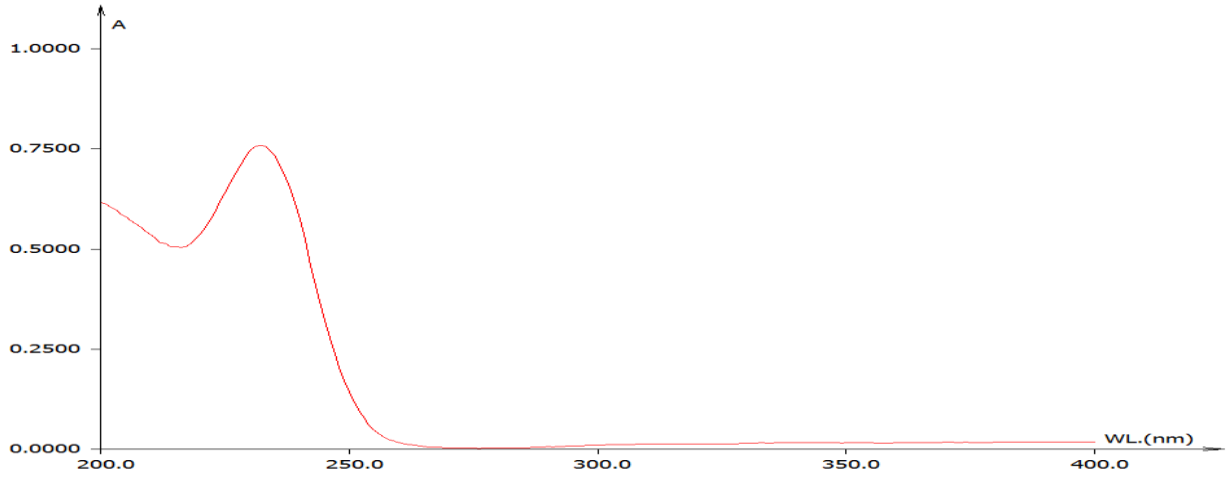

Fig 2: UV Spectrum for Tivozanib

While scanning the Tivozanib solution we observed the maxima at 235nm. The UV spectrum has been recorded on T60-LAB INDIA make UV – Vis spectrophotometer model UV-2450.

Summary of Optimized Chromatographic Conditions

The Optimum Chromatographic conditions [24] obtained from experiments can be summarized as below:

Table 4: Summary of Optimised Chromatographic Conditions

Mobile phase	ACN : Methanol: 0.1% OPA = 60:30:10
Column	Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm
Column Temperature	Ambient
Detection Wavelength	235 nm
Flow rate	1.0 ml/ min.
Run time	06 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase

Injection Volume	10µl
Type of Elution	Isocratic
Retention time	2.570 minutes

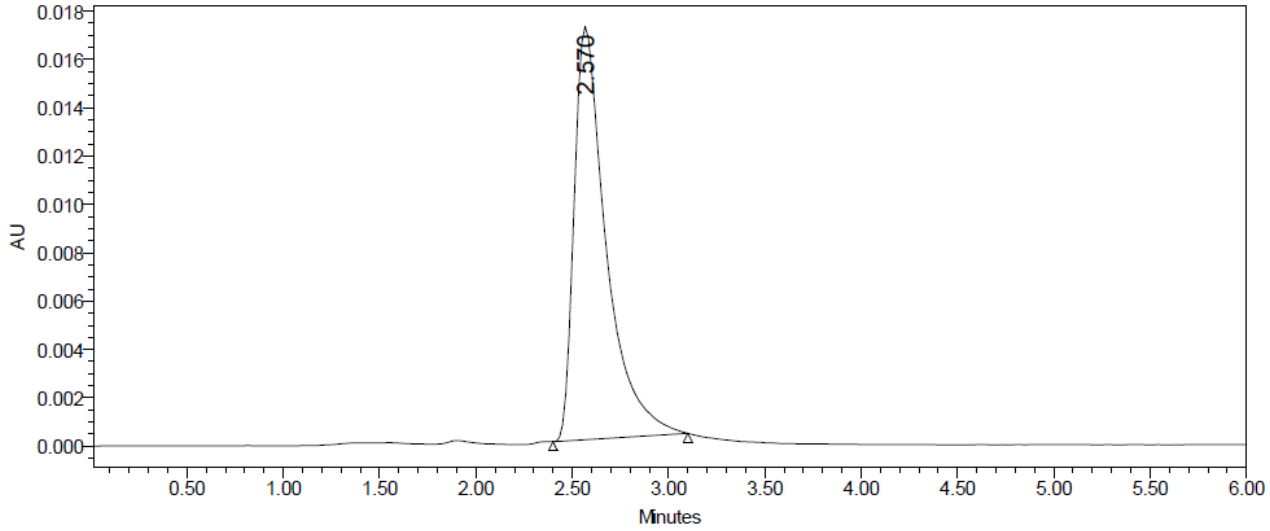

Fig 3: Chromatogram of Tivozanib in Optimized Condition

The selected and optimized mobile phase was ACN: Methanol: 0.1% OPA = 60:30:10 and conditions optimized were flow rate (1.0 ml/minute), wavelength (235nm), Run time was 06 mins. Here the peaks were separated and showed better resolution, theoretical plate count and symmetry. The proposed chromatographic conditions were found appropriate for the quantitative determination of the drug.

Validation of Method

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 Tivozanib were taken and 3 replications of each has been injected to HPLC system. From that percentage recovery values [25] were calculated from the linearity equation y = 19423x + 5444.4. The results were shown in table-5.

Table 5: Readings of Accuracy

Conc. In ppm	Conc. Found	Peak Area	% Recovery
8	8.035	161523	100.437
8	8.153	163815	101.912
8	8.061	162023	100.762
		Avg.	101.037
		S.D	0.775
		%RSD	0.767046
Conc. In ppm	**Conc. Found**	**Peak Area**	% Recovery
10	9.930	198315	99.30
10	10.033	200320	100.33
10	10.044	200540	100.44
		Avg.	100.0233
		S.D	0.628835
		%RSD	0.628688
Conc. In ppm	**Conc. Found**	**Peak Area**	% Recovery
12	11.981	238151	99.841
12	12.066	239819	100.55
12	12.215	242712	101.791
		Avg.	100.7273

	S.D	0.987021
	%RSD	0.979894

Precision

Repeatability

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Tivozanib (API). The percent relative standard deviation [26] was calculated for Tivozanib are presented in the table-6.

Table 6: Readings of Repeatability

HPLC Injection Replicates of Tivozanib	Retention Time (Minutes)	Peak Area (AUC)
Replicate – 1	2.572	197236
Replicate – 2	2.570	197762
Replicate – 3	2.573	195969
Replicate – 4	2.570	194724
Replicate – 5	2.574	198327
Replicate – 6	2.573	198711
Average		**197121.5**
Standard Deviation		**1515.213**
% RSD		**0.768667**

The repeatability study which was conducted on the solution having the concentration of about 10µg/ml for Tivozanib (n =6) showed a RSD of 0.768667% for Tivozanib. It was concluded that the analytical technique showed good repeatability.

Intermediate Precision/Ruggedness

Intra-Day & Inter-Day

The intra & inter day variation [27] of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Tivozanib revealed that the proposed method is precise.

Intra Day/Day-1/Analyst-1

Table 7: Results of Intermediate Precision Analyst 1 for Tivozanib

S. No.	Peak Name	RT	Area (µV*sec)	USP Plate count	USP Tailing
1	Tivozanib	2.580	206587	3102	1.16
2	Tivozanib	2.597	206859	2986	1.18
3	Tivozanib	2.581	207854	3054	1.13
4	Tivozanib	2.573	208965	3154	1.14
5	Tivozanib	2.590	206547	3157	1.12
6	Tivozanib	2.572	209865	3268	1.18
Mean			207779.5		
Std. Dev.			1381.9336		
% RSD			0.665		

Inter Day/Day-2/Analyst-2

Table 8: Results of Intermediate Precision Analyst 2 for Tivozanib

S. No.	Peak Name	RT	Area (µV*sec)	USP Plate count	USP Tailing
1	Tivozanib	2.580	215263	3215	1.17
2	Tivozanib	2.597	214235	3652	1.19
3	Tivozanib	2.581	213254	3496	1.15
4	Tivozanib	2.573	212367	3258	1.16
5	Tivozanib	2.590	213698	3365	1.17

6	Tivozanib	2.572	217456	3524	1.14
Mean			214378.8		
Std. Dev.			1791.516		
% RSD			0.835678		

Intraday and interday studies 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.

Linearity & Range

The calibration curve showed good linearity in the range of 6 – 14 µg/ml, for Tivozanib (API) with correlation coefficient (r^2) of 0.999 (Fig-4). A typical calibration curve has the regression equation [28] of y = 19423x + 5444.4 for Tivozanib.

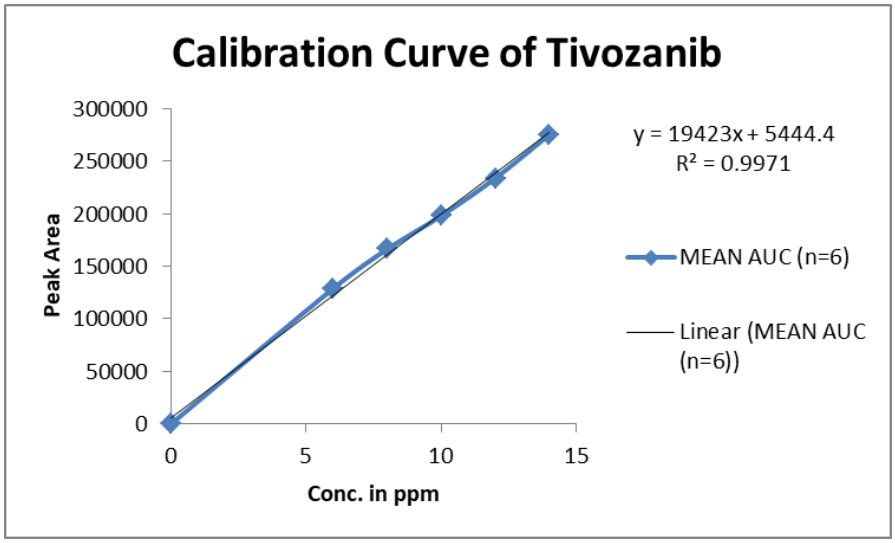

Fig 4: Calibration Curve of Tivozanib (API).

Table 9: Linearity Results

CONC.(µg/ml)	MEAN AUC (n=6)
0ppm	0
6ppm	129013
8ppm	166523
10ppm	198315
12ppm	234151
14ppm	275819

Linearity Plot

The plot of Concentration (x) versus the Average Peak Area (y) data of Tivozanib is a straight line.

Y = mx + c

Slope (m) = 19423

Intercept (c) = 5444.4

Correlation Coefficient (r) = 0.99

The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

Correlation Coefficient (r) is 0.99, and the intercept is 5444.4. These values meet the validation criteria.

Specificity

The system suitability for specificity was carried out to determine whether there was any interference of any impurities in the retention time of the analytical peak.

The study was performed by injecting blank and standard into the system. There was no interference of any peak in the blank with the retention time of the analytical peaks.

Method Robustness

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Wavelength of detection (±2nm)

& organic phase in mobile phase (±5%) studied to determine the robustness of the method are also in favour of (Table-10, %

RSD < 2%) the developed RP-HPLC method for the analysis of Tivozanib (API).

Table 10: Results for Robustness for Tivozanib

Parameter Used for Sample Analysis	Peak Area	Retention Time	Theoretical Plates	Tailing Factor
Actual Flow rate of 1.0 mL/min	203654	2.570	2915	1.16
Less Flow rate of 0.9 mL/min	265876	2.573	3652	1.19
More Flow rate of 1.1 mL/min	298653	2.631	3854	1.20
Less Organic Phase	315874	2.590	3945	1.17
More Organic Phase	326985	2.602	3487	1.19

LOD & LOQ

LOD

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.

$$LOD = 3.3 \times \sigma / s$$

Where
σ = Standard deviation of the response
S = Slope of the calibration curve

LOQ

The quantitation limit [29] of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

$$LOQ = 10 \times \sigma / S$$

Where
σ = Standard deviation of the response
S = Slope of the calibration curve
The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.08 & 0.24µg/ml respectively.

System Suitability Parameter

System suitability was carried out with six injections of solution of 100% concentration having 10µg/ml of Avapritinib in to the chromatographic system [30]. Number of theoretical plates (N) obtained and calculated tailing factor (T) was reported in table-11.

Table 11: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Asymmetry	T ≤ 2	Tivozanib=0.23
2	Theoretical plate	N > 2000	Tivozanib=2987
3	Tailing Factor	T<2	Tivozanib=1.17

Estimation of Tivozanib in Pharmaceutical Dosage Form

Each tablet contains: 1.34 mg

Twenty pharmaceutical dosage forms were taken and the I.P. strategy was taken after to decide the normal weight. Above measured tablets were at last powdered and triturated well. An amount of powder proportionate to 25 mg of medications were exchanged to 25 ml volumetric flagon, make and arrangement was sonicated for 15 minutes, there after volume was made up to 25 ml with same dissolvable. At that point 10 ml of the above arrangement was weakened to 100 ml with versatile stage. The arrangement was separated through a layer channel (0.45 □m) and sonicated to degas. The arrangement arranged was infused in five reproduces into the HPLC framework and the perceptions were recorded.

A copy infusion of the standard arrangement was additionally infused into the HPLC framework and the peak regions were recorded. The information is appeared in Table-12.

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg/tab}$$

Where:
AT = Peak Area of medication acquired with test arrangement
AS = Peak Area of medication acquired with standard arrangement
WS = Weight of working standard taken in mg
WT = Weight of test taken in mg
DS = Dilution of Standard arrangement
DT = Dilution of test arrangement
P = Percentage virtue of working standard

Table 12: Recovery Data for estimation of Tivozanib in Fotivda

Brand Name of Tivozanib	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Fotivda Tablets (Catalent CTS, Inc.)	1.34mg	1.21 (± 0.627)	99.79 (± 0.277)

The amount of drug in Fotivda Tablets was found to be 1.21 (± 0.627) mg/tab for Tivozanib & % assay was 99.79 %.

Stability Studies

The API (Tivozanib) was subjected to pressure conditions in different approaches to watch the rate and degree of debasement that is probably going to happen over the span of capacity as well as after organization to body. This is one kind of quickened strength contemplates that causes us deciding the destiny of the medication that is probably going to occur after prolonged stretch of time stockpiling, inside a brief timeframe as contrast with the continuous or long haul soundness testing. The different corruption pathways examined are corrosive (Acid) hydrolysis, essential (Base) hydrolysis, warm debasement (Thermal), photolytic corruption and oxidative debasement.

Results of Stability Studies

The results of the stress studies indicated the specificity of the method that has been developed. Tivozanib was stable in thermal and photolytic stress conditions. The result of forced degradation studies [31] are given in the following table-13.

Table 13: Results of Forced Degradation Studies of Tivozanib API.

Stress condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	81.36	18.64	100.0
Basic Hydrolysis (0.I M NaOH)	24Hrs.	83.37	16.63	100.0
Thermal Degradation (50 ^{0}C)	24Hrs.	98.92	1.08	100.0
UV (254nm)	24Hrs.	96.33	3.67	100.0
3 % Hydrogen peroxide	24Hrs.	89.41	10.59	100.0

SUMMARY AND CONCLUSION

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Tivozanib, 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 Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm Column was preferred because using this column peak shape, resolution and absorbance were good. Mobile phase & diluent for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, Acetonitrile, dichloromethane, water, 0.1N NaOH, 0.1NHCl). Tivozanib was found to be practically insoluble in water and slightly soluble, soluble in organic solvents such as DMSO and dimethyl formamide, slightly soluble in ethanol and methanol. Utilizing these solvents with suitable arrangement more current techniques can be created and approved. Discovery wavelength was chosen in the wake of examining the standard arrangement of medication more than 200 to 400nm. From the U.V range of Tivozanib it is apparent that a large portion of the HPLC works can be proficient in the wavelength scope of 210-300 nm helpfully. Further, a stream rate of 1.0 ml/min and an infusion volume of 10µl were observed to be the best investigation. The outcome demonstrates the created technique is amazingly, one more reasonable strategy for measure and dependability related debasement examines which can help in the investigation of Tivozanib in various details.

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