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METHOD DEVELOPMENT AND VALIDATION OF AN LCMS/MS METHOD FOR QUANTIFICATION OF TIVOZANIB IN RABBIT PLASMA

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Abstract
An easy, quick, precise, active and reproducible LC-MS/MS technique was developed for the bio analytical method of Tivozanib with D_4-Tivozanib as internal standard. Separation was carried on Symmetry C_{18} column (150 mm x 4.6mm, 3.5µm) using a isocratic elution with a buffer containing Hexane sulphonic acid and Acetonitrile in the ratio of 40:60 as mobile phase with 1mL/min flow rate at ambient temperature . Analysis was carried out within 5 minutes over a good linear concentration range from 50 ng/mL to 400 ng/mL (r^2 = 0.9996) for Tivozanib. Precision and recovery study results are within the acceptable limit. This method has been successfully applied, exploring Tivozanib with its internal standard (D_4-Tivozanib) was extracted from rat plasma using liquid -liquid extraction. This strategy was applied for Freeze thaw, auto sampler, bench top and long-term stability studies, we found that the drugs were stable throughout the stability studies according to USFDA guidelines.
Key words: LC-MS/MS, Tivozanib, Rabbit plasma.

INTRODUCTION

Tivozanib is used in form of the hydrochloride monohydrate, which is a white to light brown powder [1]. It is practically insoluble in water and has low solubility in aqueous acids, ethanol and methanol. It is not hygroscopic and not optically active [2, 3]. Tivozanib, sold under the brand name Fotivda, is a medication used for the treatment of relapsed or refractory advanced renal cell carcinoma (RCC) [4, 5]. It is an oral VEGF receptor tyrosine kinase inhibitor [6, 7]. The most common side effects include fatigue [8, 9], hypertension [10, 11], diarrhea, decreased appetite, nausea, dysphonia [12], hypothyroidism [13], cough, and stomatitis [14]. It should not be taken during pregnancy as it is teratogenic [15], embryotoxic and fetotoxic in rats. Administration of a single dose of tivozanib with rifampicin, a strong inducer of the enzyme CYP3A4 [16, 17], cuts the biological half-life and total exposure (AUC) of tivozanib in half, but has no relevant influence on highest concentrations in the blood. Combination with ketoconazole, a strong CYP3A4 inhibitor, has no relevant effects. A quinoline [18] urea derivative, tivozanib suppresses angiogenesis [19] by being selectively inhibitory against vascular endothelial growth factor (VEGF) [20, 21]. It is designed to inhibit all three VEGF receptors.

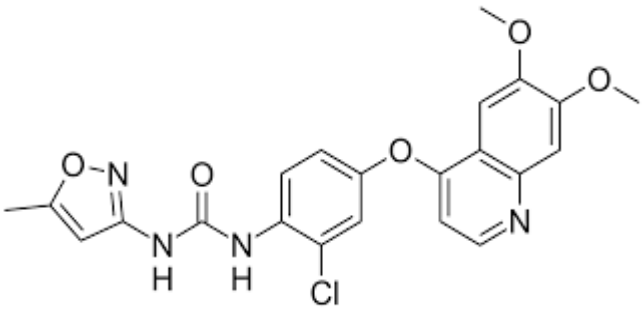

Fig. 1: Structure of Tivozanib

MATERIALS AND METHODS

Chemicals

Acetonitrile, Ortho Phosphoric acid (OPA) and water (HPLC grade), Hexane sulphonic acid were purchased from Merck (India) Ltd. Worli, Mumbai, India. All API of Tivozanib as reference standards were procured from Torrent Pharma, Ahmadabad.

Instrument and Conditions

For the development of a Bio-analytical assay, an HPLC device (Waters Alliance e2695 model) was connected to a mass spectrometer QTRAP 5500 triple quadrupole instrument (SCIEX). Chromatographic separation was achieved using a symmetry C_{18} (150 x 4.6 mm, 3.5 µm) column on an isocratic model at room temperature. The mobile step was a 40:60v/v mixture of Hexane Sulphonic acid and acetonitrile with a flow rate of 1.0 mL/min. The injection volume was 10 µL, and the total run time was 5 minutes. The analysis was carried out on a QTRAP 5500 triple quadrupole mass spectrometer with a positive ion electrospray ionisation interface. MRM mode was used to track the following mass ion pairs: m/z 510.69 for Tivozanib, m/z 514.26 for D_4-Tivozanib (Internal standard). Following are the working parameters of mass spectrometry after optimization: Ion spray voltage 5500V; temperature source 550°C; drying gas temperature 120-250°C; collision gas -Nitrogen; pressure 55psi; drying gas flow stream-5mL/min; Delustering potential 40V; Entrance potential 45V; Exit potential 15V; Capillary voltage 5500V and Dwell time 1sec.

Preparation of Standard stock solution:

Weighing 5 mg of Tivozanib into a 10 ml volumetric flask, adding approximately 7 ml of diluents, and sonicating for 15 minutes to dissolve. Then using diluents, get it up to mark. Take 1 mL of this solution and dilute it with diluents to make 10 mL. This is the parent stock of Tivozanib.

Take 0.8 mL of Tivozanib parent stock solution solution into another 10 ml volumetric flask, dilute it with diluents to make 10 mL. This is the stock of Tivozanib.

Like this prepare D_4-Tivozanib stock solution also.

Preparation of a standard solution for plasma sample

Aliquots of 200 µL of rabbit plasma specimens were spiked with 100 µL of internal normal (IS) and 100 µL of standard stock working solution. Following that, 1600 µL of acetonitrile and diluents were vortex mixed for 15 minutes, the samples were centrifuged at 5000 rpm for 15 minutes, and the supernatant handled solution was separated, collected, and filtered through a 0.45 µ nylon syringe filter into a vial before being injected into the HPLC system.

Validation of Bio analytical Method

Selectivity, Matrix Effect and Recovery

Tivozanib and its IS selectivity was tested by examining rabbit plasma specimens from 6 heaps of different rabbits for obstruction from unknown specimens at retention time. The peak zone proportion in the post extracted plasma sample from 6 separate medication free plasma samples and slick recovery samples was compared to calculate the effect matrix for Tivozanib. Trails were conducted in triplicate with six different lots of plasma at MQC levels, with a reasonable accuracy (percent CV) of 0.58%. The extraction efficiencies of Tivozanib was determined by looking at six repeats at each concentration of QC, and the degree of recovery was determined by comparing highlights of separate guidelines to non-extricated peak areas of standard.

Dilution Integrity and Carry over

Spiking matrix above the ULOQC with analyte concentration and diluting this test with a blank matrix should demonstrate dilution integrity. The analyte retained by the chromatographic device during the injection of a sample that occurs in subsequent blank or unknown samples is referred to as carry over.

Precision and Accuracy

Replication analysis of quality control specimens (n=6) was used to assess it at the lower quantification limit (LLOQ), low quality control (LQC), medium quality control (MQC), and high quality control (HQC) levels. Except for LLOQ, where CV should be less than 20%, the amount of CV should be less than 15%.

Stability

The area response of the analyte in the stability samples was compared to the region response of the specimen prepared from fresh stock solution to determine stock solution stability. Six replicates of each dose were used in plasma stability experiments at different concentration levels of LQC and HQC. According to the USFDA's guidelines, analyte was considered steady if the change was less than 15%. The stability of spiked rabbit plasma samples stored at room temperature for 24 hours was tested (bench top stability). The auto sampler stability of spiked rabbit plasma deposited in a 2-8°C auto sampler was tested for 24 hours. The auto sampler's stability was determined by comparing extract plasma samples injected immediately with samples re-injected at 2-8°C for 24 hours after storage in the auto sampler. The freeze-thaw durability was determined by comparing freshly spiked quality control samples with durability samples frozen at -30°C and thawed three times. The freeze-thaw stability test used six aliquots in each concentration spectrum in the LQC and HQC. For long-term stability testing, the concentration obtained after 24 hours was compared to the initial concentration.

Results

Bio-analytical Method development

In this step, the ESI has the most intense reaction over the chemical ionisation by atmospheric pressure (APCI) mode. The MRM mode has been used to quantify the ions of Tivozanib, and its internal standard. When compared to ion-negative mode, Tivozanib and its internal standard has a strong positive ion response mode. The details of the mass spectrum are shown in the figure 2 and 3.

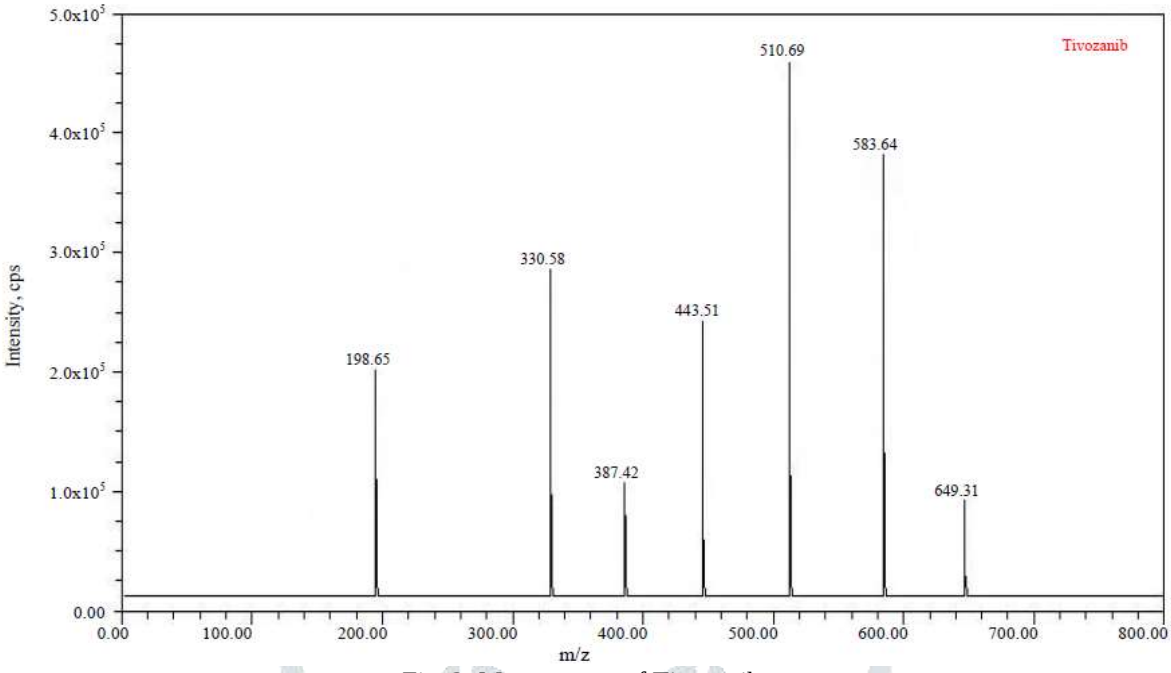

Fig. 2: Mass spectra of Tivozanib

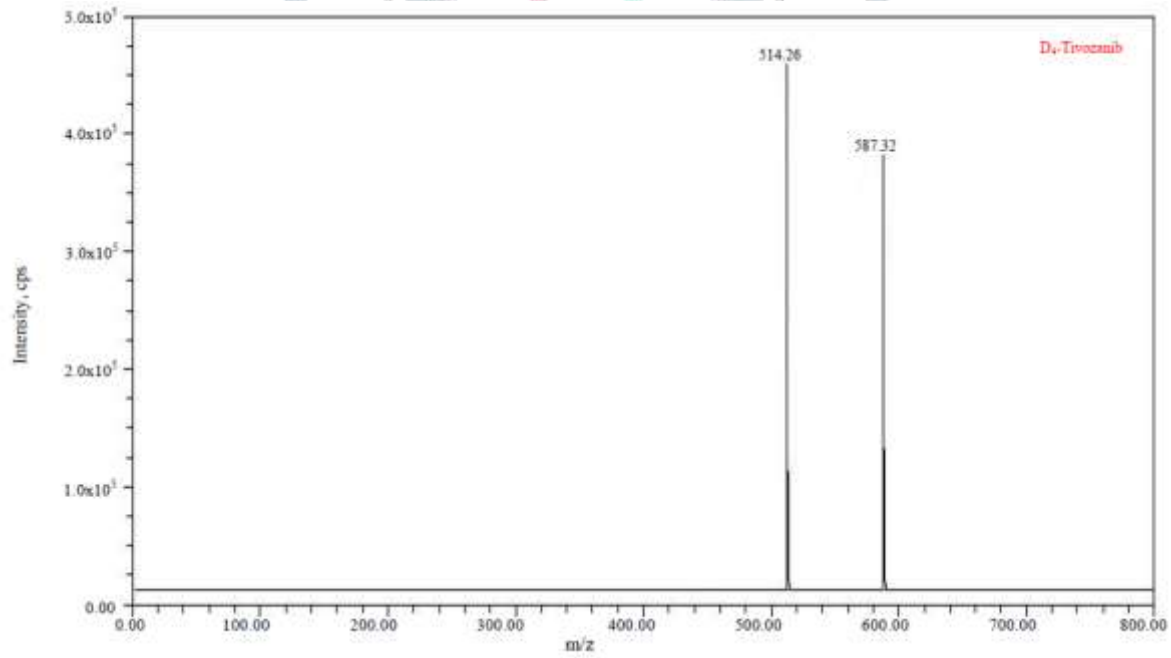

Fig. 3: Mass spectra of D_4-Tivozanib

Specificity

The specificity of the method to research Tivozanib is proved. The chromatograms of blank and standard as shown in figure 4, 5. The chromatograms of blank rabbit plasma and standard having no interference peaks were observed.

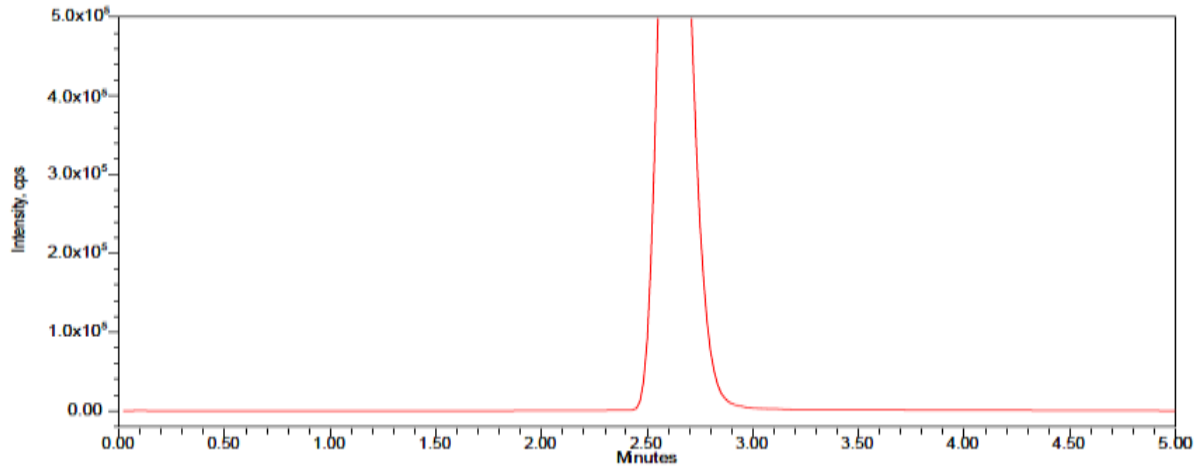

Fig. 4. Chromatogram of blank

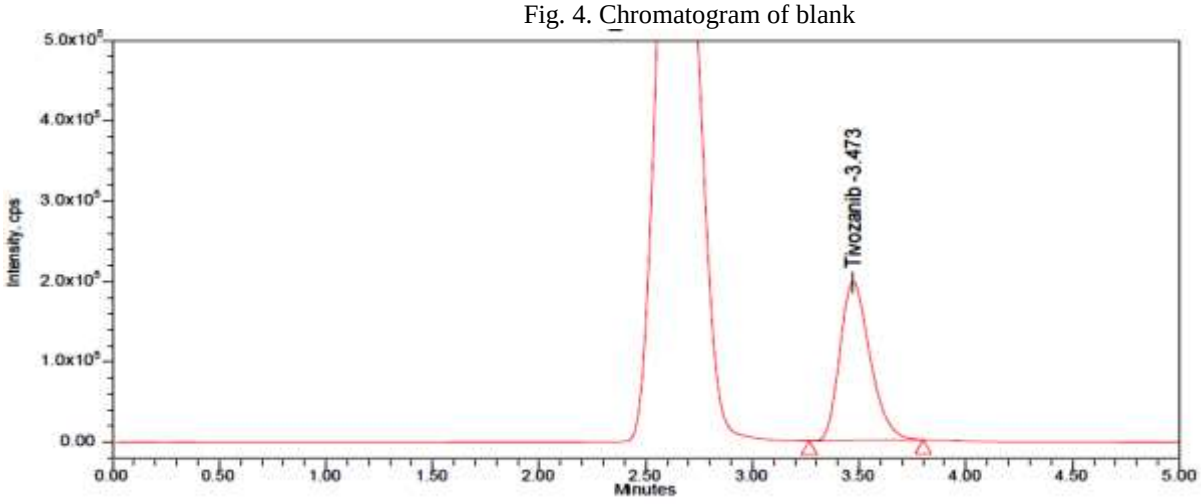

Fig. 5. Chromatogram of standard

Matrix effect

Percent RSD for within the signal, ion suppression/enhancement was observed as 1.1 percent for Tivozanib in LC-MS/MS, suggesting that under these circumstances the matrix effect on analyte ionization is within an acceptable range of ionization. In matrix effect LQC and HQC of Tivozanib were 99.13 and 97.73. %CV of the drug at LQC and HQC level were 0.69, 0.19 respectively. It indicates that the matrix effect on the ionization of the analyte is within the suitable limit.

Linearity

The peak area ratio of calibration standards was proportional to the concentration. The concentration range of Tivozanib is 50 - 400 ng/ml. Linearity results of Tivozanib was shown in following table 1 and their calibration plots were shown in figure 6. The calibration curves were appeared linear and coefficient of correlation was found to be 0.999 for Tivozanib.

Table 4. Linearity results of Tivozanib

Linearity	Tivozanib conc. (ng/mL)	Tivozanib Area response ratio
1	50.00	0.202
2	100.00	0.365
3	150.00	0.561
4	200.00	0.751
5	250.00	0.926
6	300.00	1.131
7	400.00	1.493
Slope	0.0037	
Intercept	0.01553	
CC	0.99964	

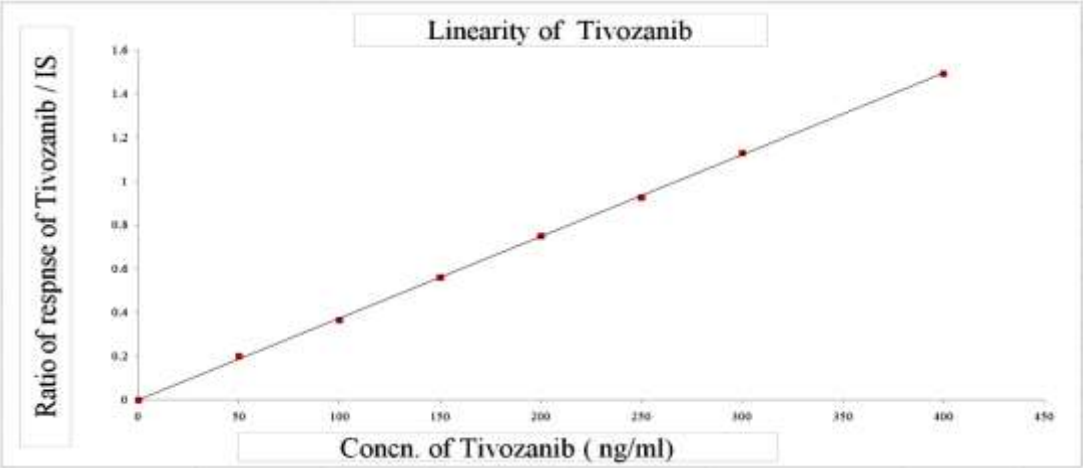

Fig. 6: Linearity plot for Tivozanib

Precision and Accuracy

By pooling all individual assay results of different internal control samples, the accuracy and precision were calculated. It was obvious, based on the data provided, that the strategy was precise and effective. The precision results of Tivozanib was shown in Table 2. Tivozanib accuracy results in quality control samples 96.32-100.05. Half of Tivozanib CV is < 5% of total internal control samples.

Table 2: Precision and Accuracy of Tivozanib

QC Name	LLQC	LQC	MQC	HQC
Conc.(ng/ml)	10 ng/ml	100 ng/ml	200 ng/ml	300 ng/ml
Mean	$0.1536x10^5$	$1.0248x10^5$	$2.0244x10^5$	$3.0175x10^5$
SD	0.00458	0.00425	0.00382	0.00129
%CV	1.02	0.38	0.58	0.64
Accuracy	96.32	98.28	100.05	99.89

Recovery

The recovery for Tivozanib at LQC, MQC and HQC levels the results demonstrated that the bio-analytical method had good extraction efficiency. This also showed that the recovery wasn't hooked into concentration. The recovery for Tivozanib (91.78% - 101.06%) at LQC, MQC and HQC levels and % CV ranged from 0.61-2.38 for Tivozanib. The results demonstrated that the bioanalytical method had good extraction efficiency.

Ruggedness

The percent recovery and percent CV of Tivozanib determined with two different analysts and on two different columns were within acceptable criteria in HQC, LQC, MQC and LLQC samples. The results proved method is ruggedness. The percent recoveries ranged from 95.85%-99.68% for Tivozanib. The %CV values ranged from 0.15-0.75for Tivozanib. The results proved method is ruggedness.

Auto sampler carryover

Peak area response of Tivozanib, wasn't observed within the blank rabbit plasma samples after successive injections of LLQC and ULQC at the retention time of Tivozanib. In auto sampler carryover this method doesn't exhibit auto sampler carryover.

Stability

Tivozanib solution was prepared with diluents for solution stability analysis and placed in a refrigerator at 2-8°C. Fresh stock solutions were associated with stock solutions that were prepared 24 hours earlier. The plasma stability of the bench top and auto sampler was stable for twenty four hours, and 24 hours at 20°C in the auto sampler. It became apparent from future stability that Tivozanib was stable at a storage temperature of -30°C for up to 24 hours. The overall stability results of Tivozanib have been stated in the below table 3.

Table 3: Stability results of Tivozanib

Stability experiment spiked plasma		Spiked plasma conc. (n=6,ng/ml)	Conc. measured (n=6,ng/ml)	%CV
Bench top stability	LQC	100	100.27	1.29
	HQC	300	300.09	0.63
Auto sampler stability	LQC	100	100.52	1.05
	MQC	200	200.65	0.73
	HQC	300	300.56	0.32
Long term (Day28)	LQC	100	100.74	0.49

stability	HQC	300	300.83	1.14
Wet extract stability	LQC	100	100.65	1.52
	HQC	300	300.48	0.74
Dry extract stability	LQC	100	100.23	0.83
	HQC	300	300.34	0.36
Freeze thaw stability	LQC	100	100.09	1.29
	HQC	300	300.18	1.48
Short term stability	LQC	100	100.43	0.89
	HQC	300	300.11	0.62

CONCLUSION

For the primary time higher sensitive HPLC-ESI-LCMS/MS method was developed and validated for the determination of Tivozanib in rabbit plasma. Here the described method is rugged, fast, reproducible bio analytical method. This method was validated according to USFDA guidelines.

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