LCMS METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF TIVOZANIB IN RAT 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 using Deuterium as internal standard. This article summarizes the recent progress on bioanalytical LC-MS/MS methods using Waters C8 (100x4.6 mm, 3.5µ) column and organic mobile phase of Ammonium formate and methanol in 70:30 ratio. The calibration curve was linear in the concentration of 50ng/ml for tivozanib. Accuracy, precision, recovery, matrix effect and stability results were found to be within the suitable limits. Simple and efficient method was developed and utilized in pharmacokinetic studies to see the investigated analyte in body fluids. The application denotes all the parameters of system suitability, specificity, linearity and accuracy are in good agreement with USFDA guidelines and applied effectively for the investigation of pharmacokinetic studies in rat.

Key words: *Tivozanib, LCMS, method development, validation, ICH guidelines.*

Introduction

Tivozanib[1,2] isanorallybioavailableinhibitor of vascular growth factorreceptors[3,4](VEGFRs) 1, 2 and 3 with potentialantiangiogenic[5] and antineoplastic activities. Tivozanib bindsto and inhibitsVEGFRs 1, 2 and 3, whichmay result in the inhibition of endothelial cell[6,7] migrationandproliferation, inhibition oftumour angiogenesis and tumourcell death. VEGFR tyrosine kinases, frequently overexpressed by a variety of tumourcell types, play a key role inangiogenesis.Tivozanib is used in form of the hydrochloridemonohydrate, whichis a white to light brown powder. It is practically insoluble inwater and has low solubility in aqueous acids, ethanol and methanol. Itis not hygroscopic[8,9]and not optically active.

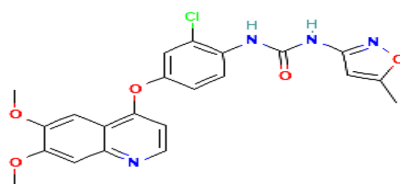

Fig 1: Tivozanib

The common side effects include fatigue [10-12],hypertension, diarrhea, decreased appetite, nausea, dysphonia, hypothyroidism, cough, and stomatitis.

1. Experimental Study

Chemicals

Acetonitrile and HSA-Hexane Sulphuric Acid, water (HPLC grade) were purchased from Merck (India)Ltd, Wroli, Mumbai, India. All APIs of tivozanib as reference standards wereprocured from Zydus Cadila, Ahamdabad

Instrumentation

An HPLC system (waters alliance e2695 model) connected with mass spectrometer QTRAP5500 triple quadrupole instrument(sciex) was used. By the Empower 2.0 software operation wasperformed [13-17].

Selection of animals:

In the following study, 6 healthy white New Zealand rats (2.0-2.5 kg) were obtainedfrom Biological E Limited, Hyderabad, India. The protocol of animal study was approved byinstitute of animal ethics committee (Reg.No:1074/PO/Re/S/05/CPCSEA).

Chromatographic conditions:

Chromatographic separation, using Waters C8 (100x4.6 mm, 3.5μ) column, was administered in isocratic mode at room temperature. As a mobile phase, a mix of Ammonium formate and methanol at 70:30 v/v with a flow of 1.0 ml/min was used. 10μl was the injection rate and the run time was 8 minutes.

Preparation of standard and internal control samples:

Preparation of standard stock solution:

Take 10mg of tivozanib working standards is taken into a 50mlvolumetric flask and add small amount of acetonitrile and small amount of hexane sulphuric acid, then sonicate for 10minutes to dissolve the contentscompletely and make up to the mark with equal amounts of acetonitrile and hexane sulphuric acid, Further dilution by taking 1.0ml into 10mlvolumetric flask. From theabove solution 0.8ml of the solution is taken into the 10ml volumetricflask and make up to the mark with the diluent.

Preparation of internal standard:

Take 5mg internal standard of tivozanib into a 10ml volumetric flask and make up to the mark with diluent and sonicate for ten minutes to dissolve the contents completely. From this solution take 1.0ml of solution into 10ml volumetric flask. From the above solution 0.8ml is taken into the 10ml volumetric flask and make up to the mark with the diluent.

Preparation of standard solution:

For standard preparation 200μl of plasma was taken and 200μl of ACN into a 2ml centrifuge tube and 100μl of standard stock solutions and 100μl of IS and 900 μl of diluents were added and vortexed for 10 min. These samples further subjected for centrifuge at 2000rpm for 30 min. Collect the solution and filter through 0.45μ nylon syringe filter and the clear solution wastransferred into vial and injected into a system.

Bio analytical Method validation:

The method was validated in selective, sensitive, linearity, accuracy and precise, matrixcondition, recovery study, re-injection reproducibility[18-19]and stability.

Selectivity:

By analyzing the six different rat plasma samples and to check interference at the retention time selectivity was conducted.

Matrix effect:

By comparing the height area ratio from the six various drug free plasma samples for tivozanib to get matrix effect. Experiments were performed at MQC levels in triplicate with six different plasma lots with the suitable precision of 15 %.

Precision and accuracy:

It was determined by replicate analysis of internal control samples at a lower limit of quantification (LLOQ), low quality control (LQC), medium quality control (MQC), top quality control (HQC) levels. The half of CV should be less than 15% and accuracy should be with in 80-120% except LLOQ where ≤20%.

Recovery:

The analysis of six samples reproduce at each internal control concentration is by extracting thetivozanib. By comparing the height areas of extracted standards to the height areas of unextracted standards, recovery is evaluated.

Carry over:

Carry over deals with the analyte retained by the chromatographic system during the matrix with an analyte [20] concentration ULOQC and above the diluting this sample with blank matrix.

Dilution integrity:

By spiking the matrix with an analyte concentration above the ULOQC and diluting this sample with blank matrix, the dilution integrity should be explained.

Stability:

By comparing the act of stock solution stability under the stability sample with the sample from the fresh stock sample preparation. Sample Stability studies in plasma were performed at LQC and HQC concentration levels using six replicates at each level. Analyte was considered stable if the change is smaller amount than 15 % as per US FDA guidelines. The perfectness[21] of spiked rat plasma stored at room temperature was evaluated for 24hrs. The stability of spiked rat plasma stored at RT in auto sampler was evaluated for 24 hrs. The auto sampler stability (LQC,MQC and HQC) was evaluated by comparing the extract plasma samples that were injected immediately, with the samples that were re-injected after storing with wet extract stability at room temperature after 12 h and 18 h at 2-8^{0}C . There injection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately, with the samples that were re injected after storing in the dry extract stability at room temperature after 12 h and 18 h at -20 ^{0} ± 30^{0}C. the freeze thaw stability was conducted by comparing the steadiness samples that had been frozen at -31^{0}C and thawed 3 times, with freshly spiked internal control samples. The short term stability was conducted 7 days at 7^0 C. For long term stability evaluation the concentrations obtained after 24 hwere compared with initial concentration.

The plasma for the above experiment has been extracted from Albino[22-26] rat i.e., shown in figure 2.

Fig 2: Albino rat

2. Results and Discussion

The maximum response on air pressure chemical ionization mode selected in this method is byhaving the electro spray ionization[27]. The mobile phase flow of 10 µl/min Tivozanib are highly responsive in the positive ion mode to offer sensitivity and signal stability withcontinuous flow to electro spray ion.

Specificity:

The specificity of the method to research Tivozanib simultaneously is proved. Thechromatograms of blank and standard as shown in figure 3,4. The chromatograms of blankrat plasma and standard having no interference peaks were observed.

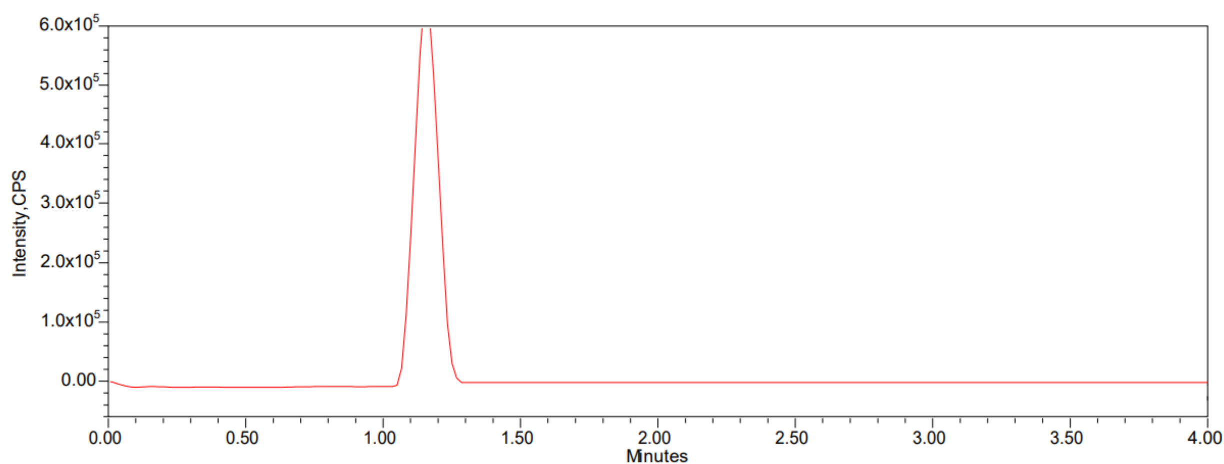

Fig. 3. Chromatogram of blank plasma

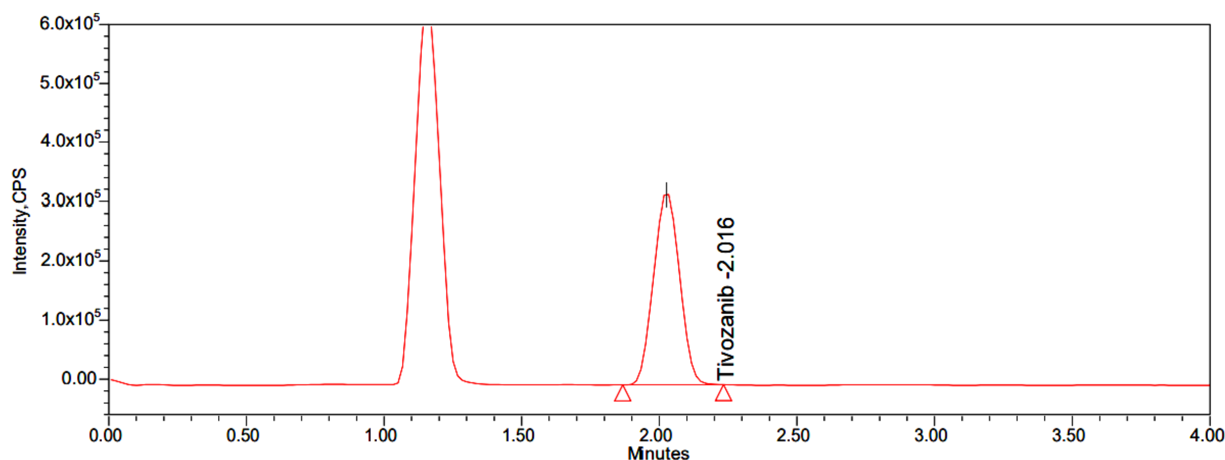

Figure 4. Chromatogram of Standard

Matrix Effect

Percent RSD for within the signal, ion suppression/enhancement was observed as 1.0 percent for in LC-MS/MS, suggesting that under these circumstances the matrix effect [28-30] on analyte ionization is within an acceptable range of ionization. In matrix effect HQC and LQC of Tivozanib were 97.73 and 99.13%. %CV of the drug at HQC level were 0.19 and LQC level is 0.69%. It indicates that the matrix effect on the ionization of the analyte is within the suitable limit.

Linearity

From the calibration curve, it was clear that the peak area ratios were proportional to the concentration. The concentration range of Tivozanib is 5-100ng/ml . The calibration curves were appeared linear and correlation coefficient was found to be 0.999 for Tivozanib. Linearity results of Tivozanib are shown in table 1.

Table 1. Linearity results of Tivozanib

Linearity	Tivozanib	
	Conc. ng/ml	Peak response
1	5.00	6259
2	12.50	16584
3	25.00	31287
4	37.50	47569
5	50.00	63254
6	62.50	79635
7	75.00	93521
8	100.00	122659
Slope	0.0231	
Intercept	0.00087	
CC	0.99967	

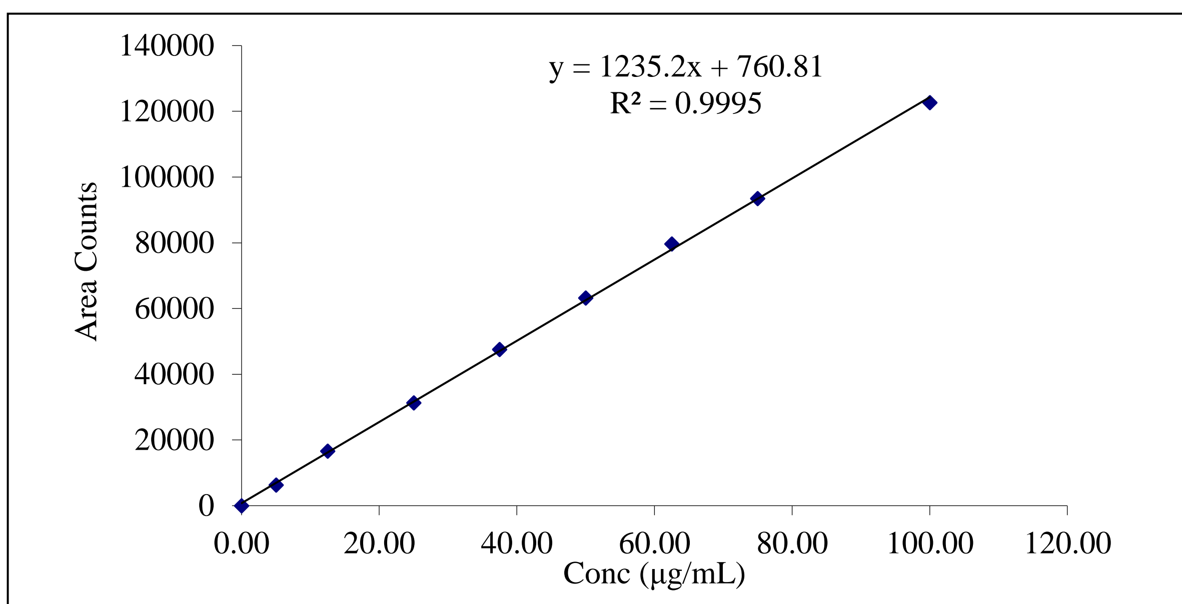

Figure 5. Calibration plot of Tivozanib

Precision and Accuracy

The intra-assay precision and accuracy were estimated by analyzing six replicates containing tivozanib at six different QC levels. The inter-assay precision [31-33] was determined by analyzing the four levels QC samples on four different runs. The criteria for acceptability of the data include, accuracy within 85–115% from the actual values and a precision of within ±15% relative standard deviation (RSD) except for LLQC, where it should be within 80–120% for accuracy and <20% of RSD

Table 2. Precision and accuracy of Tivozanib

QC Name	LLQC	LQC	MQC	HQC
Conc.(ng/ml)	5 ng/ml	25 ng/ml	50 ng/ml	75 ng/ml
QC sample -1	6235	31142	63529	93456
QC sample -2	6198	31578	63124	93740
QC sample -3	6207	31659	63857	93851
QC sample -4	6224	31027	63765	93376
QC sample -5	6189	31246	63329	93451
QC sample -6	6199	31285	63058	93652
Mean	6209	31323	63444	93588
SD	17.466	247.253	330.289	188.417
%CV	0.28	0.79	0.52	0.20
Accuracy (%)	97.37	98.24	99.49	97.84

Recovery

The recovery of drug and IS was evaluated at three concentration levels namely low, mediumand high quality control. Recovery was calculated by comparing its response in replicate samples with that of neat standard solution responses. Analyte recovery from a sample matrix (extraction efficiency) is a comparison of analytical response from an amount of analyte added to that determined from sample matrix. Because of basic properties of Tivozanib extraction was carried out using Acetonitrilesolvent. Experiments with spiked compounds resulted in recoveries of analyte 91.78 % - 101.06% and for IS 94.25 %

Ruggedness

The percent recoveries and percent CV of tivozanib determined byanalyst on a specific columnwere within acceptable criteria in HQC, LQC, MQC andLLQC samples. The results proved method is ruggedness.

Auto sampler carryover

Peak area response of tivozanib, wasn't observed within the blank rat plasmasamples after successive injections of LLQC and ULQC at the retention times of tivozanib. In auto sampler carryover this method doesn't exhibit auto sampler carryover

Stability

Tivozanib solution were prepared with diluents for solution stability analysis andplaced in a refrigerator at 2-8^{0}C. Fresh stock solutions were associated with stock solutions thatwere prepared 24 hours earlier. The plasma stability of the bench top and auto sampler wasstable for 24hours, and 24 hours at 20^{0}C in the auto sampler. It became apparent fromfuture stability that tivozanib were stable at a storage temperature of -30^{0}C for up to 24 hours. The overall stability results of tivozanib have been stated in the belowtable 5.

Table 3. Stability results of Ezetimibe

Stability experiment spiked plasma		Spiked plasma Conc(n=6,ng/ml)	Concmeasured (n=6,ng/ml)	%CV
Bench top stability	LQC	100	100.32	0.64
	HQC	300	300.45	1.73
Auto sampler stability	LQC	100	100.52263	0.89
	HQC	300	300.56567	0.525
Long term (28days) stability	LQC	100	100.436	0.29
	HQC	300	300.322	0.08
Wet extract stability	LQC	100	100.69935	1.095
	HQC	300	300.43425	0.445

Dry extract stability	LQC	100	100.284	0.99
	HQC	300	300.513	0.49
Freeze thaw stability	LQC	100	100.4543	0.6
	HQC	300	300.5132	0.17
Short term stability	LQC	100	100.415	0.37
	HQC	300	300.308	0.11

3. Conclusion

For the primary time higher sensitive HPLC-ESI-LCMS/MS method was developed and validated for the determination of tivozanib in rat plasma. Here the described method is rugged, fast, reproducible bio analytical method. This method was validated according to USFDA guidelines. Simple and efficient method was developed and may be utilized in pharmacokinetic studies and to see the investigated analyte in body fluids.

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Conflicts of Interest

Authors declare that there were no conflicts of interest.

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