IJPSR (2021), Volume 12, Issue 4 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 31 March 2020; received in revised form, 21 July 2020; accepted, 11 August 2020; published 01 April 2021

APPLICATION OF VALIDATED LC-MS/MS METHOD FOR THE QUANTIFICATION OF LENVATINIB IN BIOLOGICAL MATRICES: SUPPORTING *IN-VIVO* BIOAVAILABILITY STUDIES IN HEALTHY RABBITS

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Keywords:

Lenvatinib, Cancer, LC-MS/MS, Healthy Rabbits and bioavailability studies

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ABSTRACT: A sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique was developed and validated for the quantification of lenvatinib in plasma samples. Plasma samples were presented by selecting the Liquid-Liquid extraction (LLE) method with ethyl acetate. The technique was developed and validated with respect to the parameters specificity, sensitivity, carry-over, recovery, precision, matrix effect, accuracy, and stability. The lenvatinib and IS (lenvatinib-D4) were eluted within 3.5 min on Symmetry-C_{18} column (50 mm × 4.6mm × 5µm) with 0.1% V/V formic acid in acetonitrile, methanol, and 0.1% V/V formic acid in water in the ratio of 70:20:10 as mobile phase with 0.70 ml/min infusion flow rate. This analytical method was also applied successfully to study bio-availability studies in 6 healthy rabbits. LVB was shown T_{max} of 3.452 ± 0.754, mean C_{max} of 89 ± 3.045, AUC_{0-t} of 1700.01 ± 257.892, and AUC_{0-t} of 3665.3 ± 259.59 for Test formulation. The method can be useful in the quantification of lenvatinib in clinical research organizations for bioavailability studies and in forensic studies.

INTRODUCTION: Lenvatinib acts as an anti-cancer component useful in the treatment of certain kinds of thyroid cancers and other cancers. It inhibits the multiple kinases and acts by preventing 3 main vascular endothelial growth factor receptors (VEGFR I, II, and III), as well as platelet-derived growth factor receptor alpha, c-Kit, fibroblast growth factor receptors, and the RET proto-oncogene. These proteins mainly play an important role in cancerogenic signaling pathways. VEGFR-2 inhibition is thought to be the main reason for the most common side effect hyper-tension.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.12(4).2301-09

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(4).2301-09

LVB approved for the treatment of thyroid cancer that is locally recurrent or metastatic, progressive, and did not respond to treatment with radioactive iodine (radio-iodine). LVB is quickly absorbed from the gut, reaching peak blood plasma concentrations after 1 to 4 h (3-7 h with food).

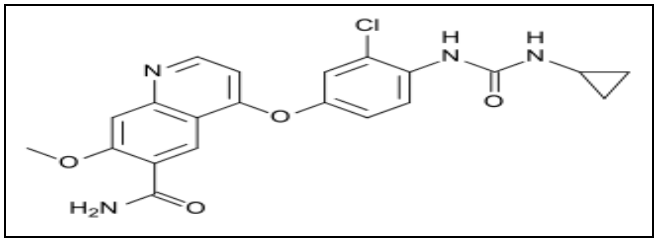


FIG. 1: STRUCTURE OF LENVATINIB

Bioavailability is found to be about 85%. The drug was almost completely (98 to 99%) bound to plasma proteins (mainly albumin). LVB is metabolized in the liver by liver enzyme CYO3A4 to desmethyl-lenvatinib (M2) [3].

The literature on LVB revealed several analytical procedures for estimating drug in formu-lations which employ high-performance liquid chromatography (HPLC) [4-7] and LC-MS/MS [8-10]. But, no method was reported on pharmacokinetic studies on healthy rabbits with low retention time.

MATERIALS AND METHODS:

Chemicals and Reagents: Lenvatinib and Lenvatinib-D4 (IS) were procured from MSN-labs, Hyderabad, Telangana, India. Water used was collected from water purification systems (Milli Q, USA) installed in the laboratory, formic acid analytical grade and were supplied by J. T. Baker, Hyderabad. The study was approved by Institutional Ethical committee no: VCP/IAEC/2016-49.

Apparatus and Software: A modular LC-system (Shimadzu, Japan) comprising of LC-20AD pump, DGU-30A4 solvent degasser, CTO-AS-column oven, and high-throughput SIL-HTC auto-sampler utilized for the work. API-4000 triple-quadrupole mass spectrometer furnished with a turbo ion-spray interface. Analyst-1.5 version software was utilized for data monitoring.

Calibration Standards: A stock solution of 1 mg/ml solution of LVB was prepared freshly by diluting 10 mg of the drug in 10 ml of 70% methanol. Calibration standards of eight different concentration levels were processed by spiking blank plasma with LVB standard solution to get the concentrations of 5.0, 20.0, 100, 250, 500, 1000, 1500, and 2000 ng/ml.

Quality Control Standards: These solutions were prepared at three different levels of lowest quality control (LQC), Median quality control (MQC), and highest quality control (HQC) standards. These Quality control (QC) samples were prepared according to calibration standards to get the concentration of 20, 500, and 1800 ng/ml for LQC, MQC, and HQC, respectively. Prepared solutions were store at -200c till the time of analysis.

Chromatographic Conditions: Isocratic Liquid Chromatographic (LC) separation was achieved on a Symmetry-C18 column (50 mmx4.6mmx5µm) with 0.1% formic acid in acetonitrile, methanol, and 0.1% formic acid in water in the ratio of 70:20:10 as a mobile phase. The LC system was operated at a flow rate of 0.70 ml/min with a total single run time of 3.5 min. The Column and auto-sampler temperatures were maintained at 40 °C and 5 °C, respectively.

Mass Instrument Conditions: The mass instrument was operated in positive ionization (PI) mode. The mass parameters were optimized based on tuning the instrument for Lenvatinib and IS by injecting the 500ng/ml solution of both drugs at a flow rate of 10 µl/min into the LC mobile phase (0.20 ml/min). Optimized mass instrument settings for specific Lenvatinib and IS where: curtain gas (CG) was 21 psi, ISG (ion source gas)-1 was 45 psi, ISG (ion source gas) 2 was 45 psi, ion spray voltage (ISV) was 5500V, turbo spray temperature at 600 ℃. Quantitative analysis was performed by multiple reaction monitoring (MRM) of the transition pairs of transitions m/z 427.10→370.10 for Lenvatinib and 430.30→370.10 for Lenvatinib D4, with 150 ms per transition as dwell time. LVB and IS precursor ions were formed by declustering potentials (DP) of 140 and 160 V, respectively, and precursor ions of LVB and IS were converted into fragments at collision energies (CE) of 30 and 30 eV with nitrogen (N2) gas at a pressure of 5 arbitrary units.

Sample Preparation Method: The sample solution was prepared by transferring 400µl of plasma and 50 µl of Lenvatinib-D4 (1 µg/ml) into a polypropylene tube and vortexed for 2 min. LVB and IS were extracted with 3ml of ethyl acetate as a solvent, followed by centrifugation of the solution at 4000 rpm/min for 15 min at 4 ºC. After centrifugation, the organic phase was separated, and it was dried using a lyophilizer. The resulting product was made solubilize in 250 µl of the mobile phase, and then it transferred into a pre-labeled vial. The vials were kept in an auto-sampler and infused into LC-MS/MS system.

Validation: Validation of the Method was processed according to Food and Drug Administration (FDA) guidelines on Bioanalytical Method Validation 11-15.

Specificity and Selectivity: The interference of analyte with endogenous matrix components was identified by close analysis of 6 batches of blank human plasma samples from different sources.

Blank and LLOQ samples were prepared for each batch and infused into the LC-MS/MS system, and results were analyzed. To measure the interference between IS and analyte, blank samples were spiked with the analyte and IS separately, and the results were evaluated. The peak area of interference peak should be <20% from the peak area of LLOQ and should be <5% from the mean IS peak area. The concentration of LLOQ should not deviate >20% from the nominal concentration (NC).

Linearity: Calibration standards(Non-zero) of eight different concentrations of 5.0, 20.0, 100, 250, 500, 1000, 1500 and 2000 ng/ml were freshly prepared and quantified in 3 separate runs. Calibration curves (peak area ratio of analyte and IS peaks versus nominal concentration) were plotted by least-squares linear regression and reciprocal of the squared concentration (1 / × 2) used as a weighting factor. Deviation should be within ± 20% for LLOQ and ± 15% for remaining concentrations [16-18].

Recovery Studies: Recovery of LVB was assessed by comparing the average peak area response of extracted samples against the unrestricted samples at the concentration levels of LQC, MQC, and HQC quality control standards. The overall mean percentage recovery was calculated from percentage recovery at each concentration level.

% Recovery of analyte = (Mean analyte peak response in extracted samples/ Mean analyte peak response in the unextracted sample) × 100

Precision and Accuracy: These parameters were assessed in the form of intraday and interday. Intra-day precision and accuracy were analyzed by infusing QC standards (20, 500, and 1800 ng/ml) and LLOQ (5 ng/ml) in five replicates a day randomly. Inter-day precision and accuracy were estimated from the analysis of each QC standard (20, 500, and 1800 ng/ml) and LLOQ QC standard (5.0 ng/ml) once a day for five different days18. The within and between batch % CV s for low, medium, and high concentrations should be within 15% except for LLOQ QC, for which the % CV should not exceed by more than 20%.

Matrix Effect: Prepare one each of LQC and HQC samples after extraction in 6 different blank matrix lots (post-extraction spiked samples). Simultaneously prepare SIX replicates of equivalent aqueous/neat QC samples and analyze. Evaluate the matrix factor for analyte and ISTD in each lot using the formula:

Matrix Factor = (Peak response in the presence of matrix ions / Average peak response in aqueous samples

Carry-over Test: Carry-over test was analyzed by infusing a sample at the upper limit of quantification (ULQC) (2000 ng/ml) and two blank plasma samples immediately after ULQC infusion. The peak response of the initial blank sample at retention time (RT) of LVB should be < 20% of the peak response of an LLOQ sample.

Stability Studies: Three sets of samples were processed without the presence of any residue extracted from blank plasma by directly spiking the analyte into the reconstitution solution. Post-preparative stability study, 3 aliquot low and high QC samples were stored at 10 ºC in an autosampler for 24 h, examined and the resulting concentrations were compared with those actual values. 3 aliquot samples of each low and high QC were stored in a deep freezer (at -70 ºC) for 36 days. The samples were examined and the resulting concentrations were compared with those actual values of QC standard samples to estimate the long-term stability of analyte. Three aliquot samples each of low and high QC (unprocessed) samples were kept at 25℃ (ambient) for 19 h in order to found the short-term stability of the analyte.

The freeze & thaw stability of the analyte was estimated after three freeze-thaw cycles at low and high QC samples. During the freeze cycle, samples were kept at -70 ºC for 24 h, and during the thaw, cycle samples were kept at room temperature. After 3 freeze-thaw cycles, the concentration of standard samples was determined.

Separate standard working solutions containing LVB and IS were prepared and kept at 25 ºC and 2 to 8 ºC for 20 h and 17 days. The peak response found from the two drugs was calculated and compared with that of the freshly prepared solutions [16].

***In-vivo* Bioavailability Studies:** Six Male healthy Rabbits weighing about 2.5 to 3 kg opted for the pharmacokinetics.

12 h before the drug administration, food was withdrawn from the rabbits until 24 h post-dosing, while water was available for rabbits throughout the study. The 1.3 mg/kg were administered to rabbits. 0.6 ml of blood samples were collected from marginal ear vein of rabbits before dosing (zero time) at the time intervals of 0.15, 0.50, 0.75, 1.00, 1.50, 2.00, 2.50, 3.50, 6.00, and 12.00 h. The resulting solution was subjected to centrifugation at 4500 rpm for 15 min, and separated plasma was transferred into labelled polypropylene tubes at -20 ºC. The protocol of the animal study was approved by the institutional animal ethics committee (IAEC NO: P62/VCP/IAEC/2017/10/DBP/AE12/Rabbits).

RESULTS AND DISCUSSION: The chromatograms of blank, High, Median and low QC samples were shown in **Fig. 2** to **5**. The developed research technique validated for the parameters linearity, specificity, sensitivity, carry-over, accuracy & precision and stability studies. The results for the validation parameters were given below.

Specificity: From the typical chromatograms shown in **Fig. 2** to **5**, no interference peaks from matrix components were detected at the retaining times of the drug sample and IS. The RT of LVB and IS were 2.653 and 2.628 min. The total elution time for LVB was 3.0 min.

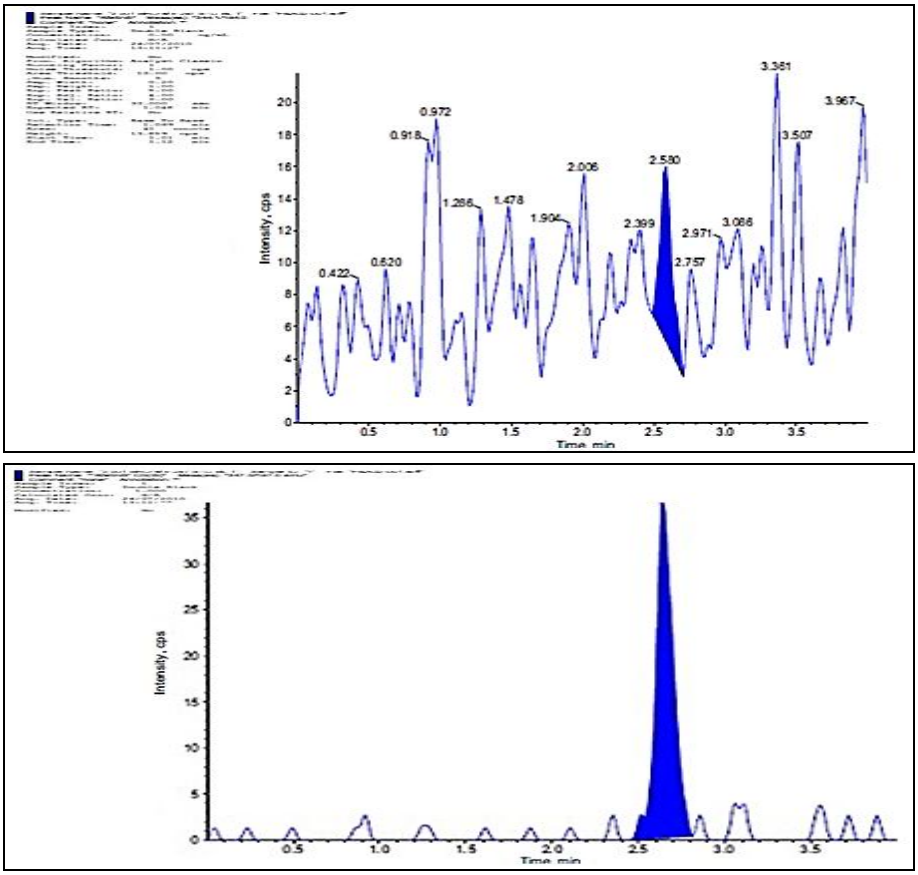
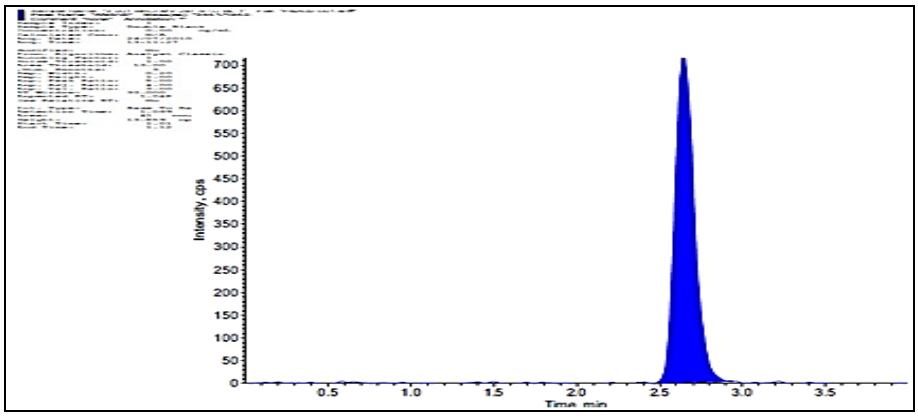


FIG. 2: CHROMATOGRAMS OF BLANK PLASMA AND IS



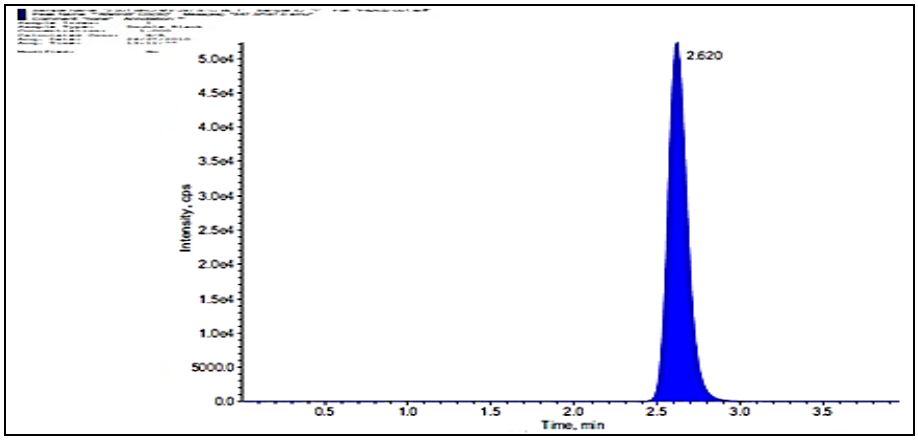


FIG. 3: CHROMATOGRAMS OF LVB & IS AT LQC LEVEL

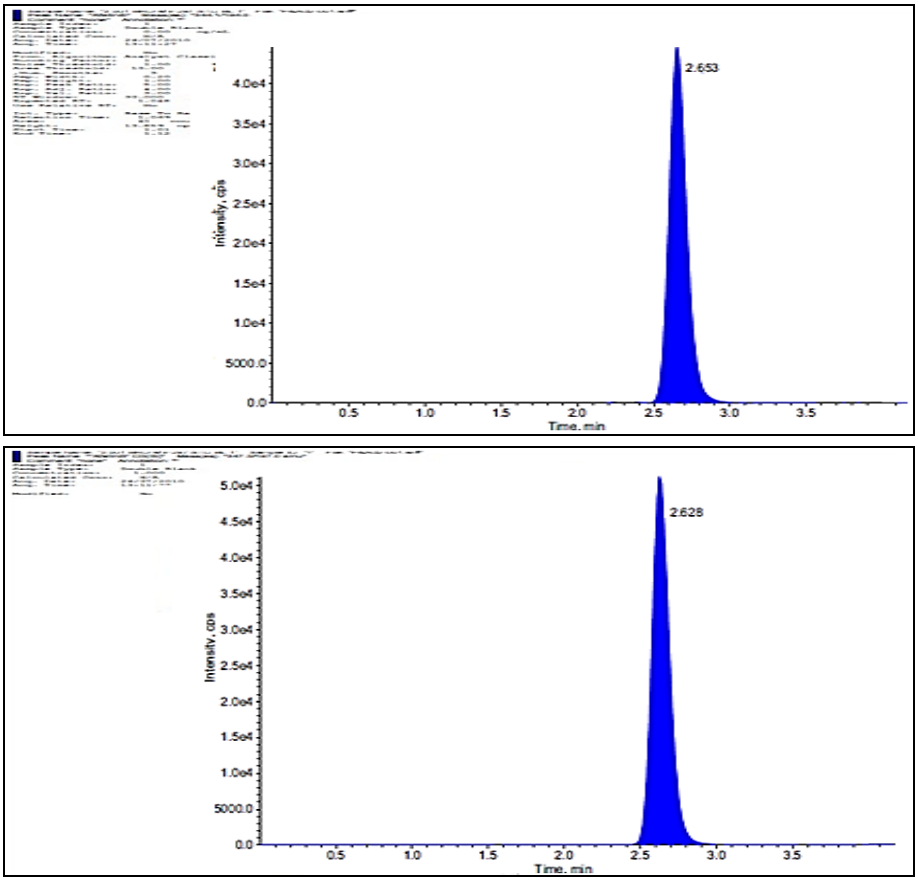
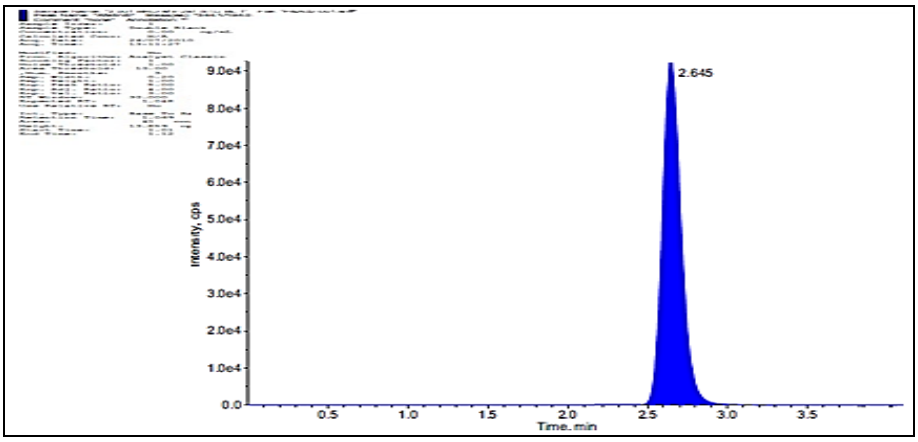


FIG. 4: CHROMATOGRAMS OF LVB & IS AT MQC LEVEL



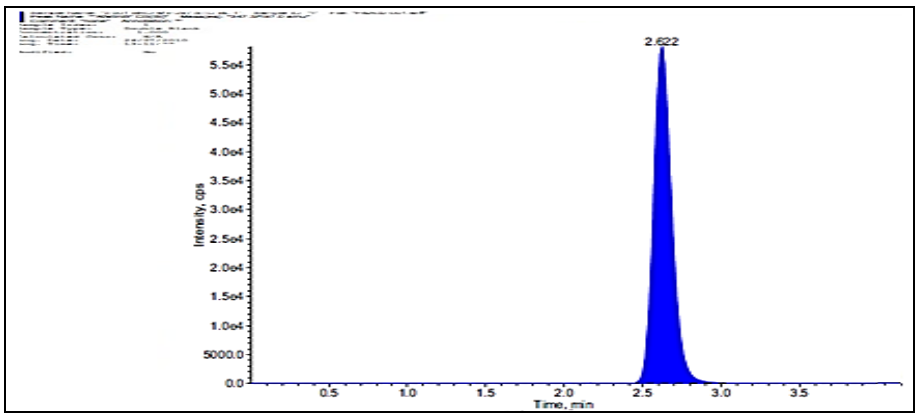


FIG. 5: CHROMATOGRAMS OF LVB & IS AT HQC LEVEL

Linearity: The calibration graphs were plotted in between the 5 to 2000 ng/ml concentration range with eight calibration standards for the analysis of linear regression. The linear regression line equation was found to be y = 0.994x + 2.662 with r2 = 0.998. The %CV measured for the calibration graph was found to be ≤ 3.73 for LVB. The findings were tabulated in **Table 1.**

TABLE 1: LINEARITY OF LVB

Actual conc. (ng/ml)	5	20	100	250	500	1000	1500	2000	Slope	Intercept
1	4.73	18.3	99.111	247.241	501.5	997.54	1547.5	1955	0.995	2.635
2	4.84	19.33	101.00	249.83	502.67	991.11	1577.50	1962.50	1.002	2.351
3	4.97	19.67	101.78	246.17	493.83	984.44	1535.00	1940.00	0.986	3.000
Mean	4.85	19.10	100.63	247.75	499.33	991.03	1553.33	1952.50	0.994	2.662
±SD	0.12	0.71	1.37	1.88	4.80	6.55	21.84	11.46	0.008	0.325
%CV	2.39	3.73	1.36	0.76	0.96	0.66	1.41	0.59		
% Accuracy	97.00	95.50	100.63	99.10	99.87	99.10	103.56	97.63		

SD- standard deviation; CV- coefficient of variance.

Recovery: The average percentage recovery of Lenvatinib at low, median, and high QC samples were found to be 90.70%, 86.49%, and 85.86%, respectively, and the results were tabulated in **Table 2.**

Precision and Accuracy: Precision and accuracy were analyzed by determining the average intra-day and inter-day precision of the analytical technique at the QC samples.

All the values were within the acceptance limit of precision, *i.e.*, 15%. The findings for precision and accuracy were represented in **Table 3.**

Matrix Effect: These parameters were calculated by determining the % CV values for High and Low QC samples and were found to be 1.64% and 0.70%, respectively. The findings were shown in **Table 4.**

TABLE 2: % MEAN RECOVERY OF LVB

	LQC			MQC			HQC		
ID	Un-extracted*	Extracted*	% Recovery	Un-extracted*	Extracted*	% Recovery*	Un-extracted*	Extracted*	% Recovery
1	0.144	0.131	90.972	0.749	0.614	81.98	1.177	1.038	88.19
2	0.137	0.126	91.971	0.763	0.628	82.31	1.217	1.024	84.14
3	0.143	0.126	88.112	0.77	0.635	82.47	1.214	0.994	81.88
4	0.143	0.133	93.007	0.715	0.64	89.51	1.248	1.046	83.81
5	0.142	0.131	92.254	0.72	0.655	90.97	1.189	1.051	88.39
6	0.149	0.131	87.919	0.725	0.665	91.72	1.173	1.041	88.75
Mean	0.143	0.130	90.706	0.740	0.640	86.493	1.203	1.032	85.861
±SD	0.004	0.003	2.184	0.023	0.018	4.705	0.029	0.021	2.939
%CV	2.69	2.27	2.41	3.17	2.88	5.44	2.38	2.02	3.42

Peak area ratio; SD- standard deviation; CV- coefficient of variance

TABLE 3: INTRA-DAY AND INTER-DAY QC SAMPLES FOR LENVATINIB

Lenvatinib				
Intra Batch	**LLOQ (5 ng/ml)**	**LQC (20 ng/ml)**	**MQC (500 ng/ml)**	**HQC (1800 ng/ml)**
Average	5.93	20.10	489.38	1844.12
SD	0.05	0.67	11.24	17.47
%CV	0.88	3.33	2.30	0.95
Mean	6.18	17.03	503.18	1887.87
SD	0.05	0.56	10.79	43.88
%CV	0.84	3.31	2.14	2.32
Mean	5.65	19.35	499.85	1830.65
SD	0.05	0.60	9.84	38.09
%CV	0.92	3.08	1.98	2.07
Inter-Batch	**LLOQ (5 ng/ml)**	**LQC (20 ng/ml)**	**MQC (500 ng/ml)**	**HQC (1800 ng/ml)**
Average	5.76	19.84	506.45	1798.98
SD	0.05	0.15	5.68	7.61
%CV	0.90	0.78	1.12	0.42

SD- standard deviation; CV- coefficient of variance.

TABLE 4: MATRIX EFFECT RESULTS FOR LENVATINIB

S. no.	LQC	HQC
	20 ng/ml	**1800 ng/ml**
1	19.782	1807.541
2	19.884	1796.917
3	19.683	1780.391
4	20.0751	1784.818
5	19.906	1730.813
6	19.982	1811.015
Mean	19.885	1785.249
± SD	0.140	29.271
% CV	0.70	1.64
% Accuracy	99.43	99.18

SD- standard deviation; CV- coefficient of variance

Stability Studies: From the result of stability studies, there was no significant degradation of analyte occurred during the chromatographic technique, extraction, and also sample storage of LVB plasma samples at different storage conditions. Stability data were represented in **Table 5.**

TABLE 5: STABILITY OF LVB IN HUMAN PLASMA

Storage Conditions	Concentration (ng/ml) Added	CV (%)	Variation (%)
Post preparative (24 h at 10 ℃)	20	2.78	-5.65
	1800	1.05	-4.45
Short-term (19 h at 25 ℃)	20	0.91	-6.98
	1800	0.66	-4.05
Long-term (36 day at -70 ℃)	20	1.22	-0.54
	1800	0.75	-2.16
Three freeze/thaw (3 cycles)	20	5.67	0.48
	1800	0.56	1.26
Stock solution (20 h at 25 ℃)	20	3.55	2.45
	1800	8.76	-1.78
Stock solution (17 day at 2 ~ 8 ℃)	20	4.49	-1.57
	1800	1.50	-0.53

CV- coefficient of variance

Application of Pharmacokinetic Study: The Pharmacokinetic parameter of LVB was calculated from the graph obtained by taking plasma concentration on Y-axis and time on X-axis using Pk-solver software. In this study, trapezoidal rule was considered for the calculation of area under the curve from 0 to 48 h (AUC0-48). LVB was shown T_{max} of 3.452 ± 0.754; mean C_{max}, AUC0□ t and AUC0□ □ for Test formulation is 89 ± 3.045; 1700.01 ± 257.892 and 3665.3 ± 259.59 respectively. The findings were shown in **Table 6, Table 7,** and **Fig. 6.**

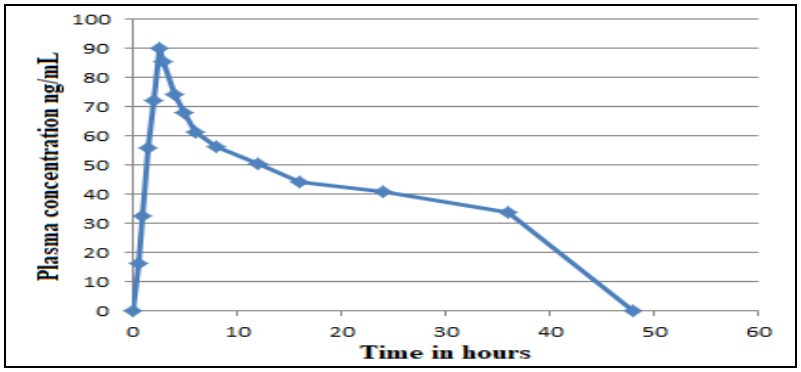


FIG. 6: MEAN PLASMA CONCENTRATION TIME PROFILE CURVE

TABLE 6: PLASMA CONCENTRATIONS AT DIFFERENT TIMINGS

Time Points in h	Measured Plasma Concentrations (ng/ml)							
	R- 1	R- 2	R- 3	R- 4	R- 5	R- 6	Avg	STDV
0	0	0	0	0	0	0	0	0
0.5	17	19	18	13	16	16	16.48	2.61
1	35	28	33	31	36	33	33.45	2.85
1.5	51	57	61	59	51	55	56	3.35
2	72	82	78	69	64	69	72.33	6.54
2.5	85	97	92	88	95	88	91	4.30
3	77	84	88	82	88	94	85.41	5.75
4	67	76	75	71	75	82	74.33	4.68
5	64	57	72	66	69	76	67.83	7.21
6	59	53	65	62	63	67	61.42	4.58
8	54	61	60	56	53	50	56.17	4.57
12	53	55	58	49	48	41	50.45	5.65
16	48	54	50	42	36	36	43.18	7.74
24	45	48	44	40	34	32	40.75	7.80
36	33	37	37	32	31	36	33.63	3.45
48	0	0	0	0	0	0	0	0

Where R indicates Rabbit; STDV- Standard deviation; Avg-Average

TABLE 7: TEST ANIMALS PK- PARAMETERS MEAN VALUES

Parameters	R- 1	R- 2	R- 3	R- 4	R- 5	R- 6	Mean	STDV
C_{max}	82	91	95	93	89	84	89	4.65
T_{max}	2.7	2.8	4.9	4.1	3.89	3.18	3.452	0.68
$t_{1/2}$	41.52	51.23	50.98	44.65	49.25	50.23	47.97	3.63
Lambda_Z	0.016579	0.016106	0.013751	0.010808	0.010912	0.023751	0.015318	0.0043
AUC_{0-t}	1745	1859.2	1654.2	1589	1498.2	1854.5	1700.01	133.15
AUC_{0-inf_obs}	3779	4198	3582	3432	3056	3945	3665.3	366.86

Where R indicates Rabbit; STDV- Standard deviation.

CONCLUSION: A simple and specific validated LC-MS/MS technique was developed for the quantitation of Lenvatinib, which provides high sensitivity with the LLE procedure. The developed analytical technique was validated with respect to the parameters specificity, sensitivity, carry-over, recovery, precision, matrix effect, accuracy, and stability. The drug and IS were eluted within 3.5 min on Symmetry C_{18} column (50 mm × 4.6 mm × 5 µm) with 0.1% formic acid in acetonitrile, methanol, and 0.1% formic acid in water in the ratio of 70:20:10 as an isocratic mobile phase with a flow rate of 0.70 ml/min.

This analytical method was also applied successfully to study bio-availability studies in healthy rabbits. LVB was shown T_{max} of 3.452 ± 0.754; mean $C_{max,}$ AUC_{0-t} and $AUC_{0-\infty}$ for Test formulation is 89 ± 3.045; 1700.01 ± 257.892 and 3665.3 ± 259.59 respectively.

ACKNOWLEDGEMENT: Authors are thankful for the Dean and Head, and co-research scholars, faculty of pharmacy Mewar University, Chittorgarh, Rajasthan, India, for their constant encouragement and support for the completion of this work. Authors are also thankful to the Central

and departmental library of pharmacy and Mewar University, Chittorgarh, Rajasthan, India, for providing necessary books and journals.

CONFLICTS OF INTEREST: All the authors were declared no conflicts of interest.

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How to cite this article:
Kanth LM and Kamal BR: Application of validated lc-ms/ms method for the quantification of lenvatinib in biological matrices: supporting *in-vivo* bioavailability studies in healthy rabbits. Int J Pharm Sci & Res 2021; 12(4): 2301-09. doi: 10.13040/IJPSR.0975-8232. 12(4).2301-09.