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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF LENVATINIB BY RP-HPLC METHOD

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Keywords:

Lenvatinib, Methotrexate, RP-HPLC, Method development, ICH Guidelines, Validation

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ABSTRACT: A rapid, fast, accurate, and precise method was developed for the estimation of lenvatinib in human plasma using methotrexate as an internal standard by reverse-phase high-performance liquid chromatography (RP-HPLC). The separation was carried out by using zodiasil C18 (150 × 4.6 mm, 5 m) as a stationary phase and 0.01 N sodium dihydrogen phosphate (pH: 4.8): acetonitrile in the ratio of 45:55 % v/v. The analysis was performed at a flow rate of 1.0 ml/min using PDA detector at 240 nm. The column temperature was maintained at 30 °C for better separation and resolution. The retention time of lenvatinib was found to be 4.508 min. The % coefficient of variation of lenvatinib was found to be 2.66%. The % recovery was found to be 94.758%. The method was found to be linear between the concentration range of 28-1120 ng/mL (r^2 = 0.999). The lower limits of quantification were 28 ng/mL, which reach the level drug possibly found in human plasma. Further, the reported method was validated as per the ICH guidelines and found to be well within a suitable range. In the future, this method can be used for clinical and pharmacokinetic studies.

INTRODUCTION: Thyroid cancer is the 6th most common cancer in women. It is the most common cancer in women aged 20 to 34. About 2% of cases occur in children and teens. Lenvatnib is an oral anti-cancer drug that is mainly used to treat radioiodine-refractory differentiated thyroid cancer [1]. Lenvatinib belongs to the class of quinolines which is the carboxamide of 4 - {3 -chloro - 4 (cyclopropylcarbamoyl) amino phenoxy} - 7 - methoxyquinoline - 6 - carboxamide [2]. Lenvatinb drug is approved in the year 2018 [5, 6].

A literature review reveals that very few analytical methods have been reported for the determination of lenvatinib by UPLC [3], RP-HPLC method [4, 7]. However, a literature review reveals that no method is reported for the determination of lenvatinib in human plasma [8] by RP-HPLC. Hence, a precise, sensitive, accurate, selective, reproducible, and rapid analytical technique for the estimation of lenvatinib in human plasma is developed and validated as per ICH guidelines [9]. The chemical structure of lenvatinib is shown in **Fig. 1**.

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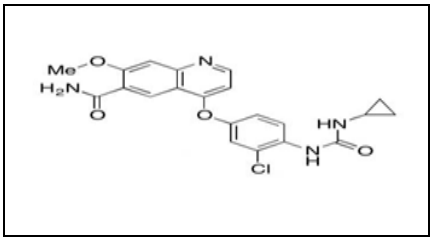

FIG. 1: CHEMICAL STRUCTURE OF LENVATINIB

MATERIALS AND METHODS:

Reagents and Chemicals: The drug sample of lenvatinib and methotrexate was obtained from BMR pharma and chemicals (Hyderabad, India).

All reagents and solvents were of analytical and HPLC grade including acetonitrile (HPLC Grade), water (HPLC Grade), potassium dihydrogen phosphate, methanol (HPLC grade), were from Rankem, Lab Chemicals, Haryana.

Instrumentation and Chromatographic Conditions: The HPLC system used was watered HPLC 2695 system equipped with quaternary pumps, photodiode array detector, and autosampler integrated with empower 2 software. Zodiacal (150 mm × 4.6mm, 5mm) column was used for separation.

The mobile phase was 0.01 N sodium dihydrogen phosphate pH (4.8) acetonitrile 45:55 (v/v). The flow rate was maintained at 1.0 ml/min, and the effluent was monitored at 240 nm.

Preparation of Solutions:

Preparation of Lenvatinib Stock Solution (140 µg/ml): Take 14 mg of lenvatinib in 100 ml volumetric flask and make the volume with diluent to produce 140 µg/ml.

Preparation of Lenvatinib Spiking Solutions (0.028 µg/ml to 11.2 µg/ml): From the above lenvatinib stock solution 0.05 ml, 0.1 ml, 0.15 ml, 0.6 ml, 1.0 ml, 1.2 ml, 1.6 ml and 2.0 ml was pipette and transferred to 8 individual 25 ml volumetric flask and make up the volume up to the mark with diluents to produce 0.28 µg/ml, 0.56 µg/ml, 0.84 µg/ml, 2.24 µg/ml, 5.60 µg/ml, 6.72 µg/ml, 8.96 µg/ml and 11.2 µg/ml.

Preparation of Calibration and Quality Control Samples: Calibration standards and quality control (QC) samples were prepared by spiking blank plasma with working stock dilutions of analytes to produce 28 ng/ml, 56 ng/ml, 84 ng/ml, 336 ng/ml, 560 ng/ml, 672 ng/ml, 896 ng/ml and 1120 ng/ml.

Preparation of Internal Standard Solution (Methotrexate):

Stock-1: Take 5 mg of methotrexate in 100 ml volumetric flask and make up the volume with diluents to produce 50 µg/ml.

Stock-2: From the above solution, take 1 ml of solution into 10 ml volumetric flask and make up the volume with diluents to produce 5 µg/ml solutions.

Note: From the above solution, take 0.5 ml of solution and spiking blank plasma with working stock dilutions of analytes to produce 1 µg/ml internal standard concentration.

Extraction Procedure: Take 750 µl of plasma and 500 µl of internal standard, 250 µl of lenvatinib from the spiking solutions of both into a centrifuging tube and add 1 ml of acetonitrile go for cyclomixer for 15 sec. Then vertex for 2 min and finally centrifuge for 5 min at 3200 rpm speed. After the centrifugation collect the sample and filter it by using polyvinylidene fluoride 0.45 µ filter and directly inject 10 µL into HPLC.

RESULTS AND DISCUSSION:

Method Development: Chromatographic conditions used was stationary phase zodiasil (150 mm × 4.6 mm, 5 mm), mobile phase 0.01N sodium dihydrogen phosphate pH (4.8): acetonitrile 45:55 (v/v) and flow rate was maintained at 1.0 ml/min and detection wavelength was 240 nm, injection volume of 10 µl and column temperature was set to 30 °C. The retention time of lenvatinib and methotrexate was found to be 4.508 min and 2.278 min respectively. The chromatogram of lenvatinib obtained by optimized conditions is shown in **Fig. 2.**

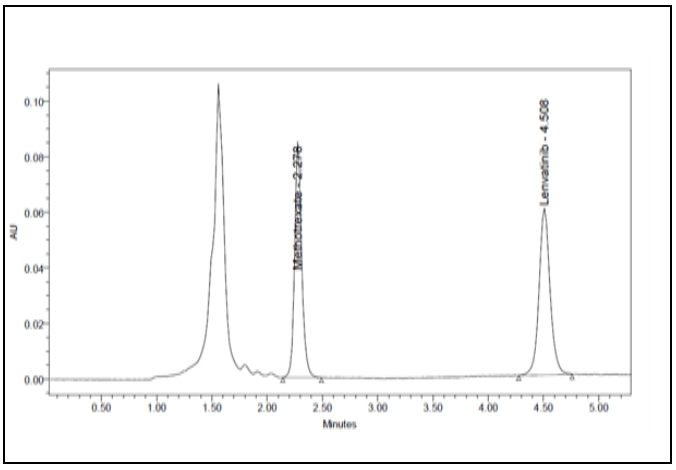

FIG. 2: OPTIMIZED CHROMATOGRAM

Bio-analytical Method Validation: The analytical method developed for the estimation of lenvatinib is validated as per ICH guidelines.

System Suitability: All the system suitability variables were within the range as per ICH rules. The % coefficient of variation for system suitability analysis was in the range of 0.04-0.46 for retention time (RT) and 0.72% for the area ratio (analyte area/IS area). The results were reported in **Table 1.**

Selectivity/Specificity: To establish the selectivity of the method, possible interference at the retention time of lenvatinib and internal standard due to endogenous plasma components were checked during the validation. Selectivity was performed by testing six batches of K2 EDTA blank plasma and the mass detection of extracted blank plasma gave good selectivity of both drug and internal standard. No interferences were found at the retention times of analytes and internal standards.

Representative chromatograms of standard blank and blank with an internal standard sample using pooled plasma are represented in **Fig. 3**, **4**, **5**, **6**.

TABLE 1: SYSTEM SUITABILITY OF LENVATINIB

Sample no.	Analyte area	Analyte RT (min)	ISD area	ISD RT (min)	Area ratio
1	18363	4.665	28049	2.242	0.6547
2	18104	4.668	27996	2.242	0.6467
3	18236	4.711	28180	2.243	0.6471
4	18401	4.710	28067	2.244	0.6556
5	18224	4.705	28292	2.243	0.6441
6	18186	4.708	28069	2.244	0.6479
Mean		4.695		2.243	0.64935
SD		0.0218		0.0009	0.004668
% CV		0.46		0.04	0.72

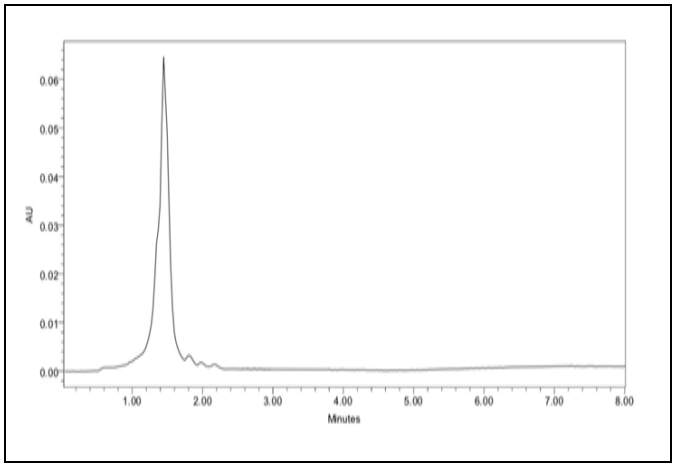

FIG. 3: CHROMATOGRAM OF EXTRACTED STANDARD BLANK SAMPLE

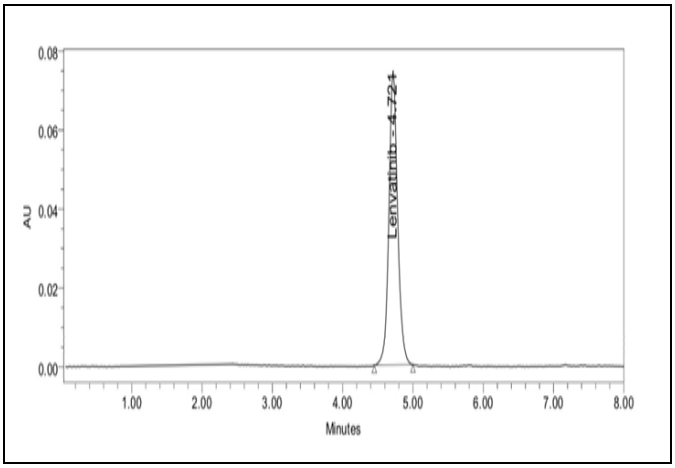

FIG. 4: CHROMATOGRAM OF PURE DRUG (LENVATINIB)

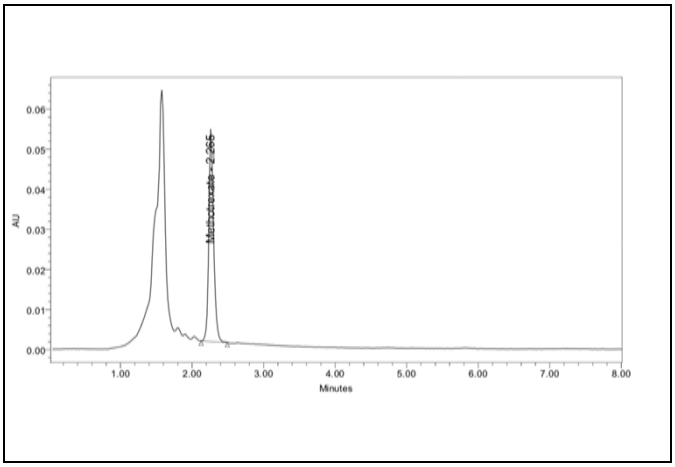

FIG. 5: CHROMATOGRAM OF METHOTREXATE (INTERNAL STANDARD)

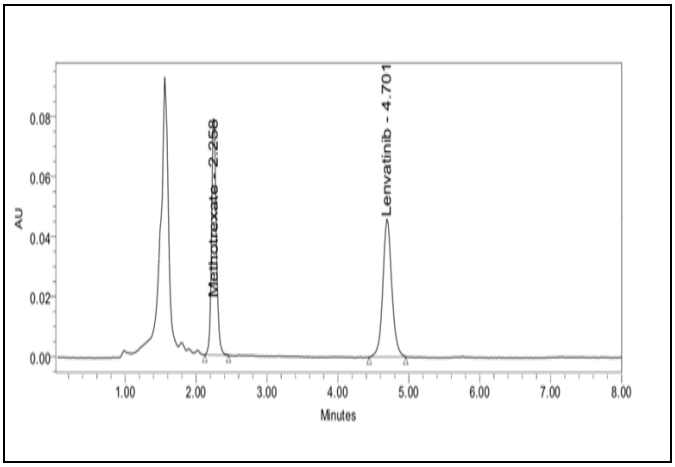

FIG. 6: CHROMATOGRAM OF ASSAY

Linearity: Calibration was found to be linear over the concentration range of 28 to 1120 ng /ml for lenvatinib. The coefficient of correlation (r^2) value was found consistently greater than 0.999 in all the cases. This indicates a high linearity of results and an excellent correlation between peak area ratios for each concentration of analytes. A representative calibration curve is shown in **Fig. 7**, which is obtained during the third precision and accuracy batch. Back calculated concentrations obtained for 3 calibration curves are summarized in **Table 2**.

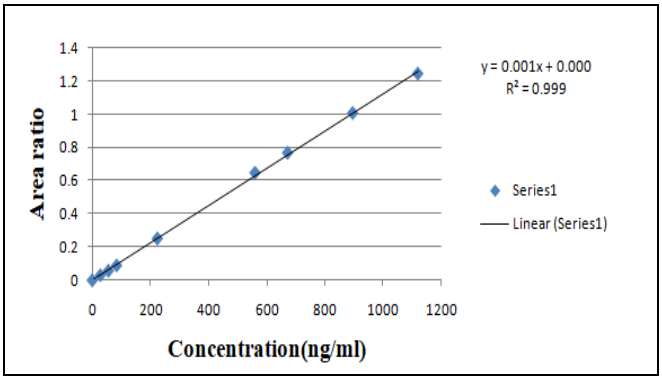

FIG. 7: CALIBRATION CURVE OF LENVATINIB

TABLE 2: LINEARITY OF LENVATINIB

S. no.	STD1	STD 2	STD3	STD 4	STD 5	STD 6	STD 7	STD 8
	Nominal concentration (ng/ml)							
	28.000	56.000	84.000	224.000	560.000	672.000	896.000	1120.000
	0.06 Nominal concentration range (ng/m1)							
	(22.400-33.600)	(47.600-64.400)	(71.400-96.600)	(190.400-257.600)	(476.000-644.000)	(571.200-772.800)	(761.600-1,030400)	(952.000-1,288000)
1	27.200	55.800	79.800	221.000	568.000	681.000	899.000	1142.000
2	26.900	58.210	83.000	225.300	552.000	675.000	885.000	1115.000
3	29.100	57.230	87.200	226.000	560.000	672.000	901.000	1166.000
Mean	27.7333	57.0800	83.3333	224.1000	560.000	676.000	895.0000	1141.0000
SD	1.19304	1.21198	3.71124	2.70740	8.00000	4.58258	8.71780	25.51470
% CV	4.30	2.12	4.45	1.21	1.43	0.68	0.97	2.24
% Mean accuracy	99.05	101.93	99.21	100.04	101.56	100.00	99.89	101.88

Precision and Accuracy: The intraday and inter-day accuracy and precision were assessed by analyzing six replicates at five different QC levels like LLOQ, LQC, MQC and HQC. Accuracy and precision method performance was evaluated by determined by six replicate analyses for lenvatinib at four concentration levels, *i.e.*, 84 ng/ml (LQC), 560 ng/ml (MQC) and 896 ng/ml (HQC).

TABLE 3: ACCURACY AND PRECISION DATA FOR INTRA-DAY RUNS OF LENVATINIB

S. no	HQC	MQC	LQC	LLOQ
	(Nominal concentration (ng/ml)			
	896.000	560.000	84.000	28.000
	Nominal concentration range (ng/ml)			
	761.600-1,030.400	476.000-644.000	71.400-96.600	22.400-33.600
1	892.790	558.210	82.751	24.263
2	894.380	555.520	80.730	27.220
3	897.980	565.320	84.680	30.260
4	895.750	564.580	87.699	28.221
5	906.890	568.900	85.780	31.251
6	903.390	574.260	90.810	29.281
Mean	898.5300	564.4650	85.4083	28.4173
SD	5.50836	6.86285	3.57888	2.48659
% CD	0.61	1.22	4.19	8.75
% Mean accuracy	100.28	100.80	101.68	101.49
1	894.340	555.160	80.790	24.281
2	892.260	558.216	82.765	26.210
3	896.120	565.020	84.720	28.255
4	898.020	564.550	87.680	32.260
5	903.940	562.650	86.640	30.243
6	901.800	560.540	90.821	29.230
Mean	897.7467	561.0227	85.5693	28.4132
SD	4.45458	3.83537	3.59469	2.85543
% CD	0.50	0.68	4.20	10.05
% Mean accuracy	100.19	100.18	101.87	101.48

The intra-day and inter-day accuracy of plasma samples were assessed and excellent mean % accuracy was obtained with range varied from 99.22%-101.66% for intraday and 99.10%-101.90 for inter-day respectively. The precision (% CV) of the analyte and plasma samples were calculated and found to be 0.45%-11.99% for intraday and 0.64%-11.17% for inter-day, respectively. The results are summarized in **Table 3, 4**.

TABLE 4: ACCURACY AND PRECISION DATA FOR INTER-DAY RUNS OF LENVATINIB

S. no	HQC	MQC	LQC	LLOQ
	(Nominal concentration (ng/ml)			
	896.000	560.000	84.000	28.000
	Nominal concentration range (ng/ml)			
	761.600-1,030.400	476.000-644.000	71.400-96.600	22.400-33.600
1	892.790	558.210	82.751	24.263
2	894.380	555.520	80.730	27.220
3	897.980	565.320	84.680	30.260
4	895.750	564.580	87.699	28.221
5	906.890	568.900	85.780	31.251
6	903.390	574.260	90.810	29.281
Mean	898.5300	564.4650	85.4083	28.4173
SD	5.50836	6.86285	3.57888	2.48659
% CD	0.61	1.22	4.19	8.75
% Mean accuracy	100.28	100.80	101.68	101.49
1	894.340	555.160	80.790	24.281
2	892.260	558.216	82.765	26.210
3	896.120	565.020	84.720	28.255
4	898.020	564.550	87.680	32.260
5	903.940	562.650	86.640	30.243
6	901.800	560.540	90.821	29.230
Mean	897.7467	561.0227	85.5693	28.4132
SD	4.45458	3.83537	3.59469	2.85543
% CD	0.50	0.68	4.20	10.05
% Mean accuracy	100.19	100.18	101.87	101.48

Recovery: Recovery was determined by measuring the peak areas obtained from prepared plasma samples with those extracted blank plasma spiked with standards containing the same area with a known amount of lenvatinib. The overall % mean recovery for lenvatinib was found to be 94.75%.

The recoveries obtained for lenvatinib at 3 QC concentration levels are summarized in **Table 5** respectively. The overall % mean recovery for methotrexate was found to be 94.12%. The recovery results of methotrexate are summarized in **Table 6**.

TABLE 5: RECOVERY-LENVATINIB

S. no.	HQC (area)		MQC (area)		LQC (area)	
	Unextracted response	Extracted response	Unextracted response	Extracted response	Unextracted response	Extracted response
1	29051	28049	20911	18419	2641	2535
2	29122	27964	20101	18906	2636	2583
3	29088	27823	20223	18850	2636	2546
4	28813	28601	20006	18667	2608	2492
5	29195	27816	19985	17972	2630	2499
6	29026	27784	20158	18689	2638	2503
Mean	29049	28006	20231	18584	2632	2526
SD	129.92	308.71	345.21	344.71	12.06	35.05
% CV	0.45	1.10	1.171	1.85	0.46	1.39
% Mean recovery	96.41		91.86		96.00	
Overall % mean recovery	94.758					
Overall SD	2.5178					
Overall % CV	2.66					

TABLE 6: RECOVERY OF METHOTREXATE (IS)

S. no.	Unextracted area	Extracted area
1	30123	28049
2	30038	27996
3	29510	28180
4	30118	28067
5	29786	28292
6	29614	28069
N	6	6
Mean	29864.8	28108.8
SD	266.75	107.95
% CV	0.89	0.58
% Mean recovery	94.12	

Stabilities:

Long Term Stock Solution Stability or Lenvatinib: In bench-top stability, six replicates of LQC and HQC samples (84 and 896 ng/ml) were analyzed for 9 h at room temperature on the laboratory bench. The % means stability was calculated and found to 100.33% for LQC and 101.87% for HQC respectively **Table 7**.

Matrix Samples Stability at -28 ± 5 °C and -80 ± 5 °C for 37 Days: Long term stock solution stability for the lenvatinib was determined at a concentration of LQC-HQC level after a storage period of 37 days at -28 °C and -80 °C in the refrigerator. 101.90%, 101.29% and 100.24%, 100.31 at 28 ± 5 °C and 101.31%, 99.70% and 100.39%, 100.32 at 28 ± 5 °C separately. The long term stability of lenvatinib is presented in **Table 8** and **9**.

TABLE 7: LONG TERM STOCK SOLUTION STABILITY OF LENVATINIB (ZERO DAY)

S. no.	HQC (Area ratio)	LQC (Area ratio)
	Nominal concentration (ng/ml)	
	896.000	84.000
	Nominal concentration range (ng/ml)	
	(7610600-1,030.400)	(71.400-96.600)
	Calculated concentration (ng/ml)	
1	892.160	82.790
2	894.580	80.751
3	898.340	84.720
4	897.920	87.690
5	907.550	90.640
6	903.210	86.820
n	6	6
Mean	898.9600	85.5685
SD	5.63455	3.56189
% CV	0.63	4.16
% Mean accuracy	100.33	101.87

TABLE 8: MATRIX SAMPLES STABILITY AT -28 ± 5 °C LENVATINIB (37 DAYS)

S. no.	HQC (area ratio)		LQC (area ratio)	
	Nominal concentration (ng/ml)			
	896.000	896.000	84.000	84.000
	Nominal concentration range (ng/ml)			
	(761.600 - 1,30.400)	(761.600 - 1,30.400)	(71.400 - 96.600)	(71.400 - 96.600)
	Comparison sample	Stability sample	Comparison sample	Stability sample
1	893.540	894.120	82.755	83.779
2	891.320	892.210	80.790	81.718
3	897.960	898.030	84.715	84.789
4	893.840	896.940	87.810	82.859
5	905.590	907.580	90.820	89.689
6	906.800	903.890	86.691	87.668
Mean	898.1750	898.7950	85.5968	85.0837
SD	6.58309	5.87162	3.61486	3.03162
% CV	0.73	0.65	4.22	3.56
% Mean accuracy	100.24	100.31	101.90	101.29
% Mean stability	100.07		99.40	

TABLE 9: MATRIX SAMPLES STABILITY AT – 80 ± 5 °C-LENVATINIB (37 DAYS)

S. no.	HQC (area ratio)		LQC (area ratio)	
	Nominal concentration (ng/ml)			
	896.000	896.000	84.000	84.000
	Nominal concentration range (ng/ml)			
	(761.600 - 1,30.400)	(761.600 - 1,30.400)	(71.400 - 96.600)	(71.400 - 96.600)
	Comparison sample	Stability sample	Comparison sample	Stability sample
1	892.697	895.798	82.784	80.795
2	890.200	892.345	84.830	78.779
3	897.894	898.895	86.750	82.748
4	907.325	903.285	83.821	86.668

5	903.592	900.992	90.680	84.810
6	905.062	901.725	81.715	88.696
Mean	899.4617	898.8400	85.0967	83.7493
SD	6.98931	4.10254	3.23696	3.70265
% CV	0.78	0.46	3.80	4.42
% Mean accuracy	100.39	100.32	101.31	99.70
% means stability	99.93		98.42	

CONCLUSION: The proposed method was validated as per the ICH guidelines and found to be well within the acceptable range. The method is simple, rapid, accurate, precise, and appropriate for pharmacokinetic and therapeutic drug monitoring in the clinical laboratories.

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CONFLICTS OF INTEREST: The authors declare that he/she has no conflicts of interest.

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