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Development and validation of stability indicating RP-HPLC method for quantitative estimation of levofloxacin injection 5mg/ml dosage form

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Abstract

A new analytical "reverse phase high performance liquid Chromatography (RP-HPLC) assay method has been developed for estimation of" Levofloxacin in injection phase. The separation was achieved by using column Inertsil ODS-3V (250 x 4.6mm, 5µm) mobile phase consisted of 0.05 M solution of citric acid monohydrate and 10 ml of 1.0 M ammonium acetate buffer and acetonitrile in the ratio of (85:15 v/v) . The flow rate was 1.0mL.min-1. Levofloxacin was detected using UV detector at the wavelength of 293 nm. The retention time of Levofloxacin was noted to be 11.20 min respectively. The method was evaluated as per ICH guidelines. The proposed method was found to be advantageous than the existing methods towards accuracy, reproducibility, and consistent.

Key words: RP-HPLC, Levofloxacin, Forced degradation and Validation.

Introduction

Levofloxacin hemihydrate (Figure 1) is a synthetic chemotherapeutic antibiotic of the fluoroquinolone drug class and is used to treat severe life-threatening bacterial infection or bacterial infection that have failed to respond to other antibiotic classes. IUPAC name is (S)-9-fluoro-2,3- dihydro- 3-methyl -10 - (4-methylpiperazin-1-yl) -7-oxo- 7H- pyrido[1,2,3-de]-1,4-benzoxazine – 6 -carboxylic acid.

Levofloxacin hemihydrate is highly water and organic solvents like glacial acetic acid and chloroform, sparingly soluble in methanol, slightly soluble in ethanol, and practically insoluble in ether. Levofloxacin hemihydrate is odourless drug. Methods for quantitative analysis of Levofloxacin by HPLC [1–7], by UV [8–10] spectroscopy in single as well as in combination, are available in the literature. The method was developed and validated as per ICH [11–13] and USP [14] guideline.

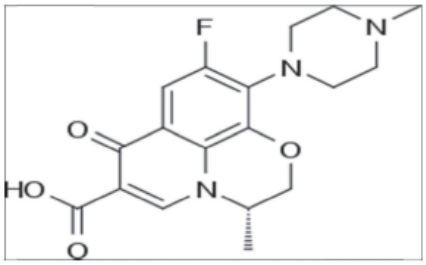

Fig.1.1. Structure of Levofloxacin

Meterials and Methods

Chemicals and reagents: Analytical-grade Ammonium acetate, Citric acid monohydrate and Hydrochloric acid was from Merck chemicals Mumbai, India. Methanol, Acetonitrile and water, both HPLC-grades, were from Merck chemicals. Mumbai, India. Millex syringe filters (0.45 ìm) were from Millex-HN, Millipore Mumbai, India.

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Instrumentation: Agilent HPLC model:1260 with DAD, Bandelin ultrasonic bath, pH Meter (Thermo Orion Model), Analytical Balance (Metller Toledo Model) were used.

Preparation of 0.05 M solution of citric acid monohydrate: Accurately weighed and dissolved 10.5 g of citric acid monohydrate in 1000 ml of water and sonicated to dissolved and mixed well.

Preparation of 1.0 M Ammonium acetate solution: Accurately weighed and dissolved 7.71 g of ammonium acetate in 100 ml of water and sonicated to dissolved and mixed well.

Preparation of buffer solution: Mixed accurately 840 ml of a 0.05 M solution of citric acid monohydrate and 10 ml of 1.0 M ammonium acetate solution.

Mobile phase: Mixed buffer solution and acetonitrile in the ratio of 85:15 v/v, filtered and degassed.

Blank preparation: Use Milli-Q water.

Standard preparation: Accurately weighed 50.0 mg of Levofloxacin working standard or reference standard was transferred into a 50 ml volumetric flask. Added 7.5ml of 0.1M Hydrochloric acid solution stirred well and diluted to the volume with 0.1M Hydrochloric acid solution. Transferred 5.0 ml of resulting solution into a 25 ml volumetric flask and diluted to volume with water and mixed well. The solution was diluted to volume with diluent and mixed well. (Concentration of Levofloxacin is about 0.2mg/ml).

Placebo solution: Transferred 10 ml of the placebo solution , added 7.5 mL of 0.1 M solution of hydrochloric acid, and diluted to volume with mobile phase and mixed well in 50 mL volumetric flask,. Further transferred 5.0 ml of the resulting solution into a 25 mL volumetric flask and diluted to volume with water and mixed well.

Sample preparation: Transferred 10 ml of the sample solution into a 50 mL volumetric flask, added 7.5 mL of 0.1 M solution of hydrochloric acid, and diluted to volume with mobile phase and mixed well.

Further transferred 5.0 ml of the resulting solution into a 25 mL volumetric flask and diluted to volume with water and mixed well.

Chromatographic conditions: Chromatographic analysis was performed on 250x4.6mm, 5µm column. The mobile phase consisted of 0.05 M solution of citric acid monohydrate and 10 ml of 1.0 M ammonium acetate buffer and acetonitrile in the ratio of (85:15 v/v). The flow rate was 1.0mL/min, column oven temperature 25°C, the injection volume was 10ìL, and detection was performed at 293 nm using a photodiode array detector (PDA).

Results and discussion

Method development: The Spectral data of compound Levofloxacin showed that maximum UV absorbance (ëmax) at 293 nm. To develop a suitable and robust LC method for the determination of Levofloxacin, different mobile phases were employed to achieve the best separation and resolution. The method development was started with Inertsil ODS-3V, 250x4.6mm, 5µm with the following different mobile phase compositions of 0.05 M solution of citric acid monohydrate buffer and acetonitrile in the ratio of 85:15 v/v. It was observed that when Levofloxacin was injected, higher retention time, Peak Tailing are not satisfactory. For next trial the mobile phase consisted of 0.05 M solution of citric acid monohydrate and 10 ml of 1.0 M ammonium acetate buffer and acetonitrile in the ratio of 85:15 v/v was employed at the flow rate of 1.0 mL/min. UV detection as performed at 293nm. The retention time of Levofloxacin is 11.20 minutes and the peak shape was good. The chromatogram of Levofloxacin standard using the proposed method is shown in **Figure: 1.2** system suitability results of the method are presented in **Table:1.2.**

Method validation: The developed RP-HPLC method extensively validated for assay of Levofloxacin using the following parameters.

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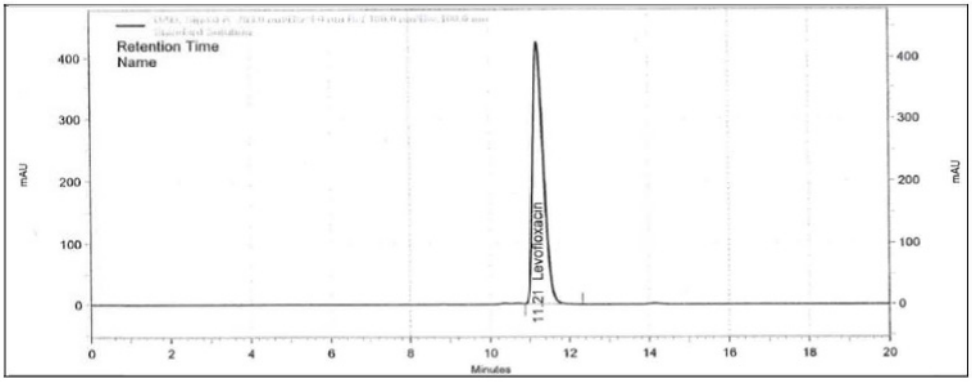

Figure 1.2. Chromatogram peak of Levofloxacin

Specificity & System suitability:

Preparation of blank solution: Used Milli-Q water as a blank solution.

Preparation of Placebo solution: Transferred 10 ml of the placebo solution into a 50 mL volumetric flask, added 7.5 mL of 0.1 M solution of hydrochloric acid, and diluted to volume with mobile phase and mixed well. Further transferred 5.0 ml of the resulting solution into a 25 mL volumetric flask and diluted to volume with water and mixed well.

Blank and Placebo interference: A study to establish the interference of blank and placebo were conducted. Diluent and placebo was injected into the chromatograph in the defined above chromatographic conditions and the blank and placebo chromatograms were recorded. Chromatogram of blank solution **Figure:1.3** showed no peak at the retention time of Levofloxacin peak. This indicates that the diluent solution used in sample preparation do not interfere in estimation of Levofloxacin in Levofloxacin injection. Similarly chromatogram of placebo solution **Figure: 1.4** showed no peaks at the retention time of Levofloxacin peak. This indicates that the placebo used in sample preparation do not interfere in estimation of Levofloxacin in Levofloxacin injection.

Table 1.1: Specificity results for Levofloxacin

S.No	Name	Retention Time (min)
1	Blank	ND
2	Placebo solution	ND
3	Standard solution	11.24
4	Sample solution	11.22

The chromatogram of blank and placebo are not showing any peak at the retention time of Levofloxacin.

Table 1.2: System suitability parameters for Levofloxacin

No.of injections	Tailing factor	Tailing plates	Theoretical Area of Levofloxacin
Inj-1	1.4	7622	1088768955
Inj-2	1.4	7580	1086835821
Inj-3	1.4	7590	1088318629
Inj-4	1.4	7527	1089977609
Inj-5	1.4	7598	1086265129
		Average %RSD	1088033229 0.14

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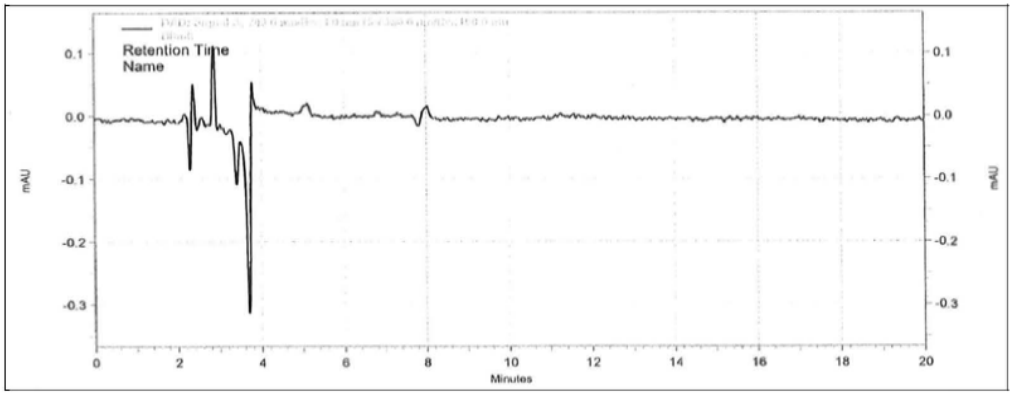

Figure: 1.3. Chromatogram showing the no interference of diluent for Levofloxacin.

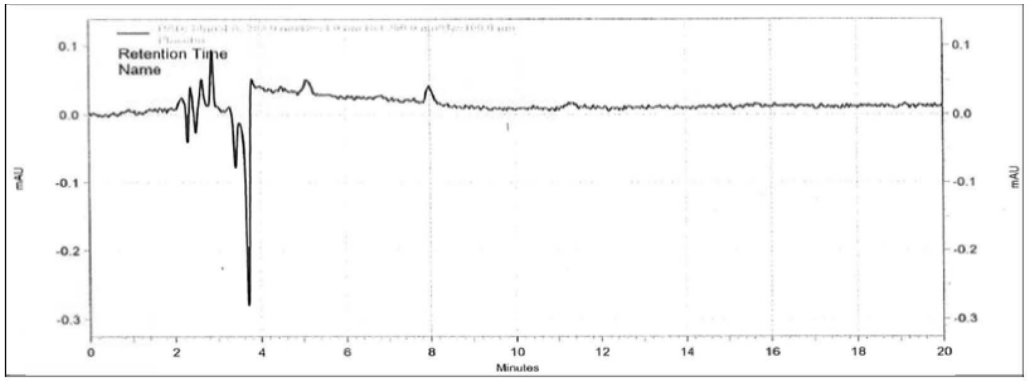

Figure: 1.4 Chromatogram showing the no interference of placebo for Levofloxacin.

Forced Degradation study: The study involves assessing the effect of acid (0.1N HCl, 2 hrs at 60˚C temperature), base (0.1N NaOH, 2 hrs at 60˚C temperature), hydrogen peroxide (3%, 2 hrs at 60˚C temperature), Thermal (105°C for 48 hours) and UV light (7days) on Levofloxacin injection samples. The chromatograms obtained from various stress conditions are shown in **Figure:1.5**. The percent assay, percent degradation and peak purity of Levofloxacin and retention time of degradants produced in all stress conditions are determined and summarized in **Table:1.3**. Levofloxacin was found to be more stable in applied acid, base, thermal and photolytic stress conditions. Levofloxacin was sensitive to adopted stress condition like oxidation. The results proved that the developed assay method has good selectivity and specificity, and is suitable for assay of Levofloxacin in the presence of stress degradation products.

Method precision: The precision of test method was evaluated by doing assay for six samples of Levofloxacin injection as per test method. The content in mg and % label claim for Levofloxacin for each of the test preparation was calculated. The average content of the six preparations and % RSD for the six observations were calculated. The chromatogram was shown in **Figure: 1.6** and data were shown in **Table: 1.4.**

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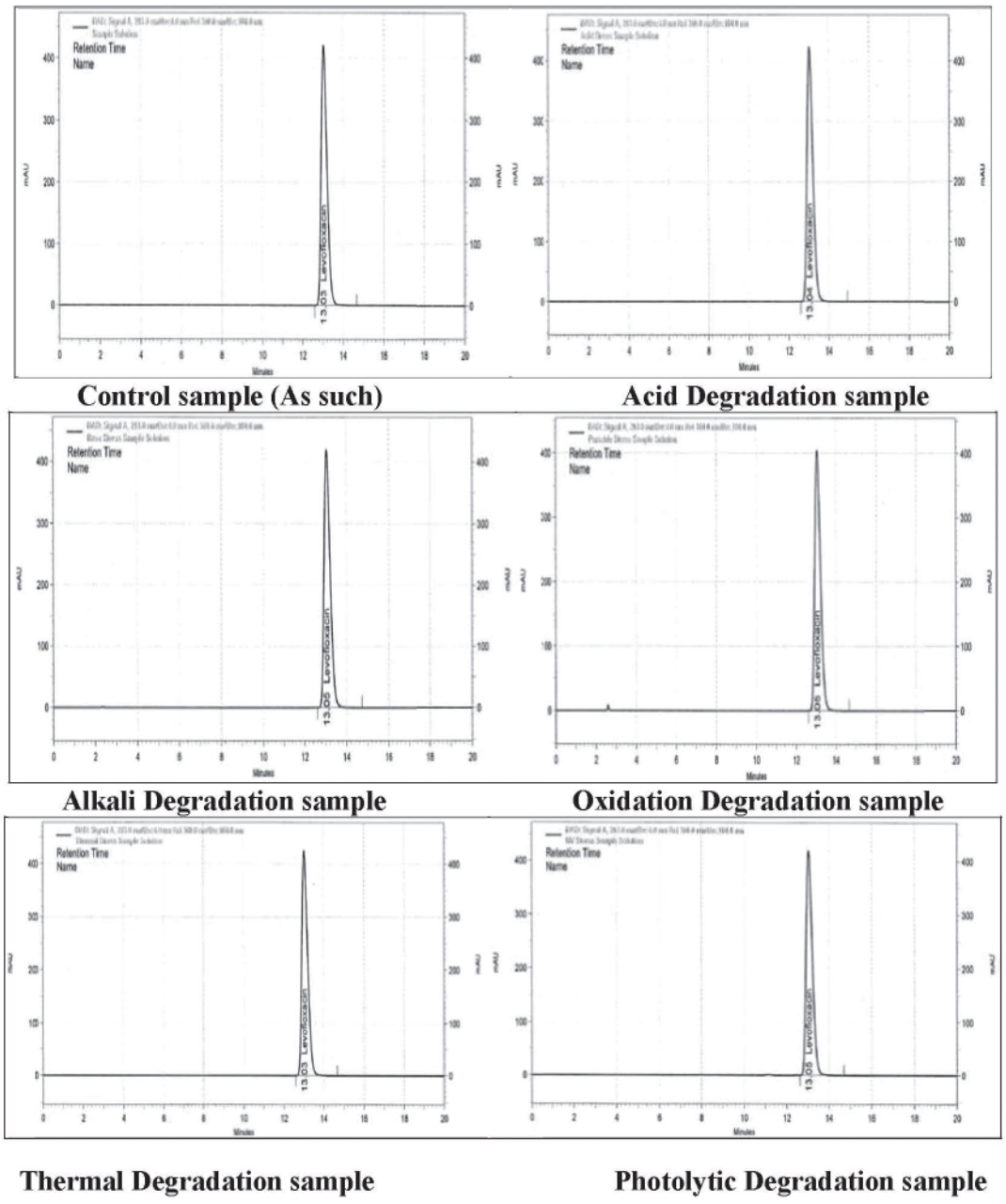

Figure: 1.5. Degradation chromatograms for Levofloxacin.

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Table: 1.3. Forced degradation results of Levofloxacin

Degradation condition	Levofloxacin % Assay	% Degradation	Peak Purity
Unstressed Sample	105.16	NA	1
0.1N HCl/60°C for 2 hours	106.47	No degradation observed	1
0.1N NaOH/ 60°C for 2 hours	105.59	No degradation observed	1
3% H_2O_2/60°C for 30 min	101.26	3.90	1
Thermal 105°C for 48 hours	106.56	No degradation observed	1
UV Light at 254nm for 7 days	104.98	0.18	

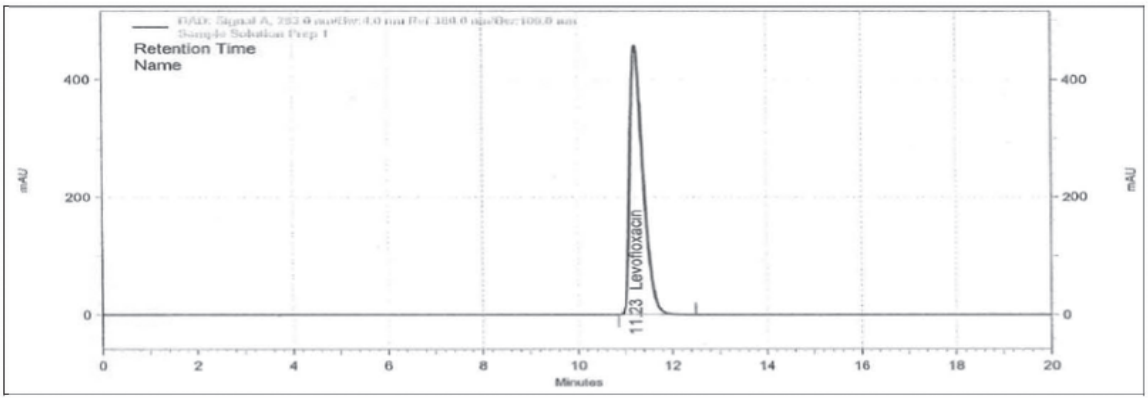

Figure: 1.6. Method precision sample chromatogram

Table: 1.4 Method precision data for Levofloxacin

No. of injections	Levofloxacin % assay
1	106.2
2	105.7
3	105.3
4	105.1
5	104.5
6	105.4
Average	**105.4**
SD	**0.57**
% RSD	**0.54**

Intermediate Precision: The intermediate precision of test method was demonstrated by carrying out method precision study in six samples, representing a single batch by two different analysts on two different days, different column, different HPLC system and by different analyst. These samples were prepared as per the test method. The % assay was calculated for each of these samples. The precision of the method was evaluated by computing the % Relative standard deviation of % assay of Levofloxacin.

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Table: 1.5 Intermediate precision data for Levofloxacin

S.No.	Area of Levofloxacin	Assay of Levofloxacin
1.	186778849	104.4
2.	187067790	104.6
3.	187523508	104.9
4.	187333783	104.8
5.	187033298	104.6
6.	186616560	104.4
	Average	**104.6**
	%RSD	**0.19**

- Overall and individual % of Assay are complies as per test method specification.
- The relative standard deviation of six assay preparations is **0.19.**
- The overall relative standard deviation of six assay preparations of precision study and six assay preparations of intermediate precision study is **0.54.**

Linearity of detector response: The standard curve was obtained in the concentration range of 100.07-300.21ìg/ml for Levofloxacin. The linearity of this method was evaluated by linear regression analysis. Slope, intercept and correlation coefficient [r] of standard curve were calculated and given in **Figure: 1.7** to demonstrate the linearity of the proposed method. From the data obtained which given in **Table: 1.6** the method was found to be linear within the proposed range.

Table: 1.6 Linearity studies for Levofloxacin by proposed method

% Level	Concentration (ppm)	Levofloxacin Area
50	100.0713	590667579
75	152.1083	900530185
100	200.1425	1157439141
125	252.1796	1461276205
150	300.2138	1737499643
	correlation coefficient	0.9999
	Slope	5705485.636
	Intercept	23004636.17
	%Y-intercept	1.99

Accuracy: The accuracy of the test method was demonstrated by preparing recovery samples of Levofloxacin at 50%, 75%, 100%, 125% and 150% of the target concentration level. The recovery samples were prepared in triplicate for each concentration level except 50% and 150 % (50% and 150% are six preparations). The above samples were chromatographed and the percentage recovery of each sample was calculated for the amount added. Evaluated the precision of the recovery at each level by computing the Relative Standard Deviation of six preparations for 50% and 150% level recovery samples results. The percentage recoveries with found in the range of 99.0 to 99.9 for Levofloxacin. The chromatogram was shown in **Figure: 1.8 to 2.2** the data obtained which given in **Table: 1.7** the method was found to be accurate.

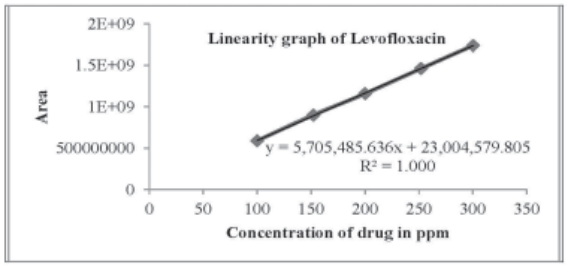

Fig. 1.7. Calibration curve for Levofloxacin

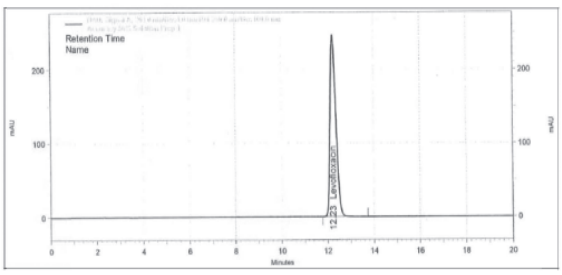

Fig. 1.8. Accuracy (Spike level 50%) chromatogram

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Table: 1.7 Recovery studies for Levofloxacin by proposed method Recovery of Levofloxacin

Sample	% Recovery	Mean % Recovery	% RSD
50% sample-1	100.9	101.0	0.27
50% sample-2	100.8		
50% sample-3	101.2		
50% sample-4	101.3		
50% sample-5	101.2		
50% sample-6	100.6		
75% sample-1	101.0	101.3	0.3
75% sample-2	101.3		
75% sample-3	101.6		
100% sample-1	101.8	101.8	0.25
100% sample-2	102.0		
100% sample-3	101.5		
125% sample-1	100.9	100.7	0.25
125% sample-2	100.7		
125% sample-3	100.4		
150% sample-1	101.5	101.4	0.28
150% Sample-2	101.7		
150% Sampe-3	101.1		
150% sample-4	101.2		
150% sample-5	101.7		
150% sample-6	101.1		

Robustness studies: To validate the method robustness the chromatographic performance at changed conditions was evaluated compared to nominal conditions of the method. Standard solution was injected at each of the following changed conditions:

Table: 1.8. Robustness studies Results

Parameter		Theoretical plates	Tailing factor	%RSD of peak area
Flow variation ±10%	10%	8599	1.4	0.07
	-10%	9820	1.5	0.12
Temperature variation ±5°C	+5°C	8467	1.3	0.16
	-5°C	8126	1.3	0.06
Mobile phase Variation± 10%	10	7622	1.4	0.10
	-10	8064	1.4	0.08

- Method is robust for changes like column oven temperature, flow rate and organic phase of mobile phase.

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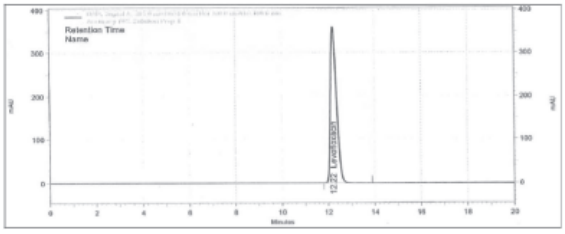

Fig. 1.9. Accuacy (Spike level 75%) chromatogram

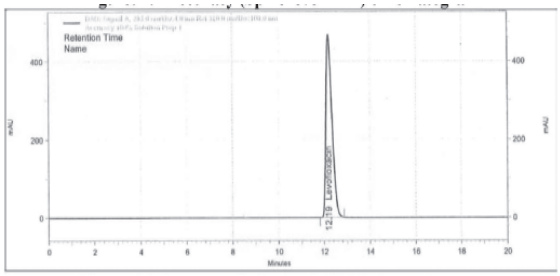

Fig. 2.0. Accuracy (Spike level 100%) chromatogram

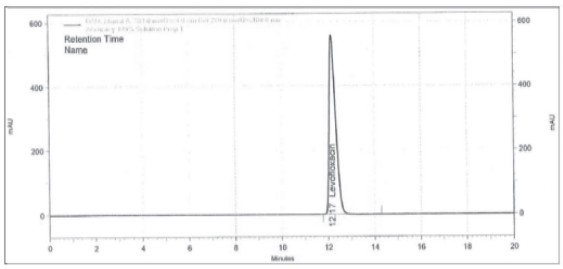

Fig. 2.1. Accuracy (Spike level 125%) chromatogram

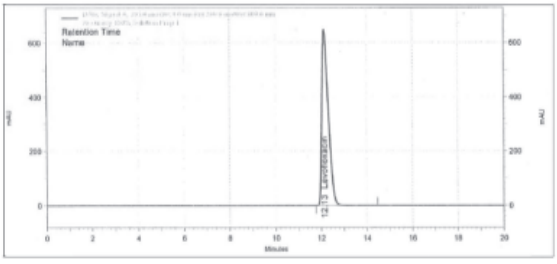

Fig. 2.2. Accuracy (Spike level 150%) chromatogram

Solution stability of analytical solutions: Levofloxacin standard and sample solutions were kept for about 48 hrs at room temperature in transparent bottles in auto sampler and in refrigerator 2-8°C. The response of these was compared with respect Initial standard solution and sample solution.

Table: 1.9. Results for solution stability of standard at room temperature

Time Interval	similarity factor
Initial	-
24hrs	1
48hrs	1

Table: 2.0 Results for solution stability of standard in Refrigerator

Time Interval	similarity factor
Initial	-
24hrs	1
48hrs	1

Table: 2.1 Results for solution stability of standard at room temperature

Time Interval	% Assay	% of Assay difference
Initial	106.2	NA
24hrs	107.52	1.32
48hrs	107.07	0.87

Table: 2.2 Results for solution stability of standard in Refrigerator

Time Interval	% Assay	% of Assay difference
Initial	106.2	NA
24hrs	107.85	1.65
48hrs	107.77	1.57

- Standard and sample solutions are stable for 48 hours when stored at room temperature (RT) and 2-8°C in refrigerator.

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Conclusion

An RP-HPLC method for estimation of Levofloxacin was developed and validated as per ICH guidelines. A simple, accurate and reproducible reverse phase HPLC method was developed for the estimation of Levofloxacin in bulk drugs and formulations. The optimized method consists of mobile phase 0.05 M solution of citric acid monohydrate and 10 ml of 1.0 M ammonium acetate buffer and acetonitrile in the ratio of (85:15 v/v) with Inertsil ODS-3V(250 x 4.6mm, 5µm) column. The retention time of Levofloxacin was found to be 11.20 minutes. The developed method was validated as per ICH Q2A (R1) guidelines. The proposed HPLC method was linear over the range of 100.07-300.21ppm, the correlation coefficient was found to be 0.9999. The percentage recoveries (accuracy) with found in the range of 99.0 to 99.9 for Levofloxacin. Relative standard deviation (%RSD) for method precision and intermediate precision was found to be 0.54 and 0.19. Solution stability of the Standard and sample solutions are stable for 48 hours when stored at room temperature (RT) and 2-8°C in refrigerator. Our developed method to be considered as fast, simple and reliable analytical method for determination of Levofloxacin in pharmaceutical preparation using RP-HPLC. As there is no interference of blank and placebo at the retention time of Levofloxacin, It is very fast with good reproducibility and response. Validation of this method was accomplished and getting results to meet all the requirements. The method is simple, reproducible, with a good accuracy and Linearity. It allows reliably the analysis of Levofloxacin in its different pharmaceutical dosage forms.

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Conflict of interests : The authors claim that there is no conflict of interest.

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