

Bio-Analytical Method Development for Estimation of Levofloxacin: Application in Estimation of Drug in Nano-formulations and Pharmacokinetic Studies

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ABSTRACT

Aim/Background: Levofloxacin is a safe antibiotic for various inflammatory disorders and a rapid analytical method for various samples reduces the estimation time as well as injects efficiency in the system. The developed method uses minimal solvents with least preparation time also helps in reducing the errors generated by the analyst during processing. **Materials and methods:** RP-HPLC method was developed for estimation of levofloxacin using Box Behnken Design. The PDA detector was set at 295nm with a run time of 6 mins. The samples were run at binary gradient mode with 35% phase B. The mobile phase consisted of acetate buffer, acetonitrile and methanol. Linearity was established for pure drug as well as spiked plasma samples. Drug extraction from plasma samples was done by liquid-liquid extraction using acetonitrile and methanol. **Results:** The developed method has a retention time of 3.1 ± 0.41 mins and is validated as per ICHQ2R1 guidelines. The standard curve indicates high correlation with a R^2 value of 0.9999. The robustness ($RSD < 2$) and system suitability studies indicate that it is a stable method with high specificity as determined with different samples. The LOD and LOQ were found to be 0.08ppm and 0.25ppm respectively indicating estimations of samples with a low drug content. **Conclusion:** A rapid, robust, selective, simple and sensitive method was developed and validated for quick estimation of levofloxacin in different sample. The significant reduction in retention time obtained by the developed method proves its efficiency in tapping the mobile phase costs as well as expediting the analytical process. The DOE optimised method is suitable for rapid estimation of levofloxacin in plasma as well as nanoformulations.

Key words: Bioanalytical method, Robustness, Specificity, Stability, ICHQ2R1.

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INTRODUCTION

Levofloxacin hemihydrate (LH) a fluoro-quinolone antibiotic effectively used to treat tubercular infections. It is the most effective broad spectrum antibiotic for gram positive and gram negative bacterial infections. LH is a Biopharmaceutical Classification System (BCS) class 1 drug with a 6-8 hr elimination half-life. Extensive literature review showed us a wide array of estimation methods of levofloxacin. Several of these were tried to estimate the drug

content in our hybrid poly D,L-lactic-co-glycolic acid (PLGA) nanoformulations as well as for pharmacokinetic studies to least efficiency. LH is a well-studied drug. Therefore, while developing the reversed phase-High performance liquid chromatography (RP-HPLC) method we came across some easy methods, some troublesome methods with advanced detection techniques and gradient elution methods. The one thing that remained

consistent throughout were the gradual improvements in estimation methods. The study is aimed to develop HPLC method that can effectively estimate LH in plasma, nanoformulations and conventional formulations as well as in cytology studies. The reduced run time and lesser chemicals required in mobile phase preparation will lead to reduced instrumental, method and personal errors due to lesser variables.

MATERIALS AND METHODS

Method development and optimization was done using design of experiments. Box Behnken design was selected as it provided greater flexibility in selection of variables and reduces the number of experimental runs. BBD helps in optimising the experimental parameters without increasing the runs and maintaining the rationality of the design

Chemicals

Levofloxacin was a generous gift from Sun Pharma. Acetonitrile, Methanol, Formic acid, orthophosphoric acid and ammonium acetate were purchased from Merck. Milli Q water was used throughout the study. All chemicals and reagents used in HPLC were of chromatographic grade.

Method Development

A box-behnken design was chosen to develop the process parameters for estimation of levofloxacin hemihydrate. 3 factors at 3 levels with 6 centre points were selected for the study. The design was selected as it reduces the number of runs of a full factorial without compromising on the effectiveness of the factors. The factors and levels selected for optimization were pH (pH 4-5), column oven temp (30-40°C) and flow rate (0.8-1.2). 18 experimental runs were performed randomly and HPLC areas, Number of theoretical plates (NTP), tailing factor and retention time were the dependant factors for the study. Experimental design matrix and the Analysis of variance (ANOVA) results obtained are tabulated in Table 1 and 2.

Chromatographic Conditions

The analysis was carried out on Shimadzu (Shimadzu corporation, Japan) equipped with a PDA detector SPD M20A with a D₂ lamp. Chromatograms were recorded by LC solutions software. Analysis was performed on Waters X Bridge C₁₈ column (250X4.6mm, 5 µm). Auto sampler SIL 20AC was used for sample injection with a needle stroke of 52mm at a sampling rate of 15µl/sec. Injection volume was set at 25µl and detection wavelength at 295 nm. Oven was maintained at 30°C

and samples were maintained at ambient temperature. Mobile phase selected for separation of components was a mixture of 100mM ammonium acetate buffer and 1:1 mix of acetonitrile and methanol. Binary gradient mode was utilized with 35% phase B at an overall flow rate of 0.93ml/min for 6 min run time.

Preparation of mobile phase

Mobile Phase A- accurately weighed amount (3.854 g) of ammonium acetate (Mettler Toledo ME 204 digital balance) was transferred to a 500ml Borosil volumetric flask, dissolved ammonium acetate in 0.1% formic acid containing Milli Q water. Diluted orthophosphoric acid was used to maintain the pH using Hanna pH meter. Mobile phase was clarified through 0.22µm membrane filter and degassed in a sonicator bath. Mobile Phase-B: a mixture of 1:1 acetonitrile and methanol respectively were used. Preparation of Diluents-1:1 Acetonitrile and methanol were used as diluents for preparation of samples which is hereafter referred to as diluent.

Preparation of Stock solutions

The standard stock solution of Levofloxacin (1000 ppm) was prepared. 10 mg of accurately weighed Levofloxacin was transferred to 10 ml volumetric flask and 5 ml of diluent was poured in it, sonicated the drug solution and made up the volume with diluent. The stock solution was transferred to 5ml volumetric flasks and half fold diluted concentrations of 128 parts per million (ppm) to 0.5 ppm were prepared for linearity studies. Estimation of limit of detection (LOD) and limit of quantification (LOQ) considered 5-point calibration curve from 0.5 ppm to 8 ppm.

Sample preparation for spiked plasma calibration curve.

Drug free plasma was spiked with a stock solution of levofloxacin to obtain a concentration of 1000 ppm. This mixture was deproteinated in presence of acetonitrile and methanol. It was vortexed for 5 mins and centrifuged at 13500 rpm for 30 mins. The clear supernatant obtained was separated and dried in a nitrogen evaporator. The dried product was reconstituted using the diluent to obtain a concentration of 512 ppm. The prepared samples were filtered through 0.22 µm nylon syringe filters. The suitable volume of the resulting solution was transferred and diluted to 128 ppm. Nine different concentrations of plasma standard samples were prepared by half-fold dilution technique i.e., 128 ppm, 64 ppm, 32 ppm, 16 ppm, 8 ppm, 4 ppm, 2 ppm, 1 ppm and 0.5 ppm for establishment of linearity and range. Similarly, five different

Table 1: Experimental design matrix and optimisation parameters.

Standard	Mobile phase pH	Flow rate (ml/min)	Column Oven Temp (°C)
1.	4.00	0.80	35.00
2.	5.00	0.80	35.00
3.	4.00	1.20	35.00
4.	5.00	1.20	35.00
5.	4.00	1.00	30.00
6.	5.00	1.00	30.00
7.	4.00	1.00	40.00
8.	5.00	1.00	40.00
9.	4.50	0.80	30.00
10.	4.50	1.20	30.00
11.	4.50	0.80	40.00
12.	4.50	1.20	40.00
13.	4.50	1.00	35.00
14.	4.50	1.00	35.00
15.	4.50	1.00	35.00
16.	4.50	1.00	35.00
17.	4.50	1.00	35.00
18.	4.50	1.00	35.00
Optimisation parameters	Goal	Lower limit	Upper limit
HPLC Area	is in range	2500000	3900000
USP (NTP)	is in range	4000	5300
Tailing	is in range	0.5	1
R.T	is in range	3	3.5

concentrations range from 0.5 ppm to 8 ppm were considered for LOD and LOQ estimation by signal to noise technique.

Preparation of sample for pharmacokinetic study

Wistar rats procured from Indian Veterinary Research Institute (IVRI), Bareilly were divided into groups as per the approved protocol. The animals were fasted overnight and were given water *ad libitum*. The animals were administered with single oral dose of developed formulation and blood samples were collected in heparinised tubes through retro-orbital route under anaesthesia. Levofloxacin was extracted from plasma sample by using liquid-liquid extraction method. The tubes were centrifuged at 13500 rpm for 10 min and plasma was separated. The plasma samples were immediately put in -20°C refrigerator. 100 µl samples were taken out from each tube for analysis and mixed with a diluent. Deproteination was followed by centrifugation and separation of clear supernatant. The clear

supernatant was dried and reconstituted with diluent. The resulting samples were filtered through 0.22 µm nylon filter prior to injecting in HPLC. The observed values were calculated through the following parameters obtained by spiked plasma calibration curve. The slope was 129255 with an intercept of -94992. The approval number for pharmacokinetic studies is BBDNIIT/IAEC/2019/01.

Preparation of nanoformulation

Soya lecithin, PLGA and levofloxacin were dissolved in dichloromethane in individual beakers. Lipidic and polymeric solutions were added to the aqueous phase containing poly vinyl alcohol under continuous stirring followed by sonication. The mixture was stirred for 24h followed by centrifugation at 10000 rpm for 30 min. The final product pellet was lyophilized and subjected to drug content estimation.

Method Validation

In house method validation for the current method was carried as per ICH Q2R1 guidelines. Validation was done through linearity, system suitability, and limit of quantification, limit of detection, range, accuracy, recovery, precision, robustness, solution stability, specificity and ruggedness

Linearity

Linearity is the ability within a specific range to obtain areas proportional to the concentration of the analyte. Working solutions were prepared from 1000ppm stock solution stored at -20°C. Suitable dilutions were prepared in triplicate for 128ppm to 1ppm. Least square linear regression equation was applied to the average area obtained from the study versus the concentration in ppm. Correlation, slope, intercept and R² values were obtained from it.

Spectrum Scan and System Suitability

System suitability studies are carried out to ensure the reproducibility and better resolution of the peaks each time the analysis is carried out. System suitability studies were done by simultaneously injecting 6 shots of similar concentrations and assessing suitability by comparing the observed HPLC area, number of theoretical plates and tailing factor.

Precision

Method precision was done by injecting 6 test solutions. Intermediate precision was done by a different analyst on different days under same experimental conditions. Standard deviation and overall relative standard deviation (RSD) were estimated.

Accuracy and recovery

Accuracy of the method is ascertained by the recovery samples. The accuracy of the method was carried out by determining the recovery at three levels in triplicate 50% (32ppm), 100% (64ppm) and 150% (96ppm).

Robustness

It is determined by making deliberate changes to the method parameters leading to reliable analytical results. Changes were made with reference to mobile phase composition, column oven temperature and flow rate. It was estimated by running 6 replicates at a concentration of 64ppm.

Solution Stability

As part of robustness of the method and stability check of the solution, a solution stability test was performed by placing a 64ppm standard solution in tightly closed volumetric flasks at room temperature and under refrigeration. They were analysed at 4hr, 12 hr and 24 hr.

Peak Purity

It is carried out for confirmatory studies to prove that the analyte peak in chromatogram is not corresponding to one or more similar components. The peak purity for the current study was determined at 32ppm.

RESULTS AND DISCUSSION

The main target of the present investigation was to develop a method which can easily and specifically separate and quantitate LH from nanoformulations, plasma solutions, marketed formulations and cytology studies. To achieve the set goal, Box Behnken Design (BBD) was chosen due to its capacity to incorporate more variables with a lesser number of runs and better design resolution. BBD is an experimental design for response surface methodology. The design is fit to be used for quadratic models and gives better results in surface plots. Design Expert 11.1.2.0 was utilised for BBD. The assumptions putative to method development were

- Less use of organic solvents to minimise cost
- Difference between the pKa values of solvent and LH
- Run time should be less than 10 mins with low retention time.
- Tailing factor remains less than 1.5.
- Maximising the number of theoretical plates

The factors selected and results obtained for BBD are enumerated in Table 1 and 2. The study of ANOVA for the different dependent variables showed up a quadratic model with significant fit for all the factors and

non-significance of lack of fit. The R^2 value for all the factors approached unity. Column oven temperature (C) played the insignificant role while mobile phase pH (A) and flow rate (B) were found as factors with significance with high F values. Mobile phase pH had insignificant effect ($p=0.0512$) on retention time (RT), whereas flow rate had insignificant effect ($p=0.1772$) on tailing. Tailing was significantly affected by mobile phase pH ($p=0.013^*$) and by the product of BC ($p=0.0054^*$). A^2 affected HPLC Area and NTP ($p=0.0108^*$ and 0.0038^* , respectively). B^2 affected tailing and RT with p values of 0.0155^* and 0.0012^* , respectively. Mobile phase pH significantly affected HPLC area and USP NTP with p values of $< 0.0001^*$ and $< 0.0001^*$ respectively. Flow rate affects HPLC area, USP (NTP) and retention time significantly with p values of 0.0003^* , $< 0.0001^*$ and $< 0.0001^*$ respectively. Based on the assumption made during method development numerical and graphical optimisations were done and solutions with high desirability values were run for confirmation of point prediction. The resulting desirability cube and overlay plot is given in Figure 1. The equations for determination of different factors are enlisted below

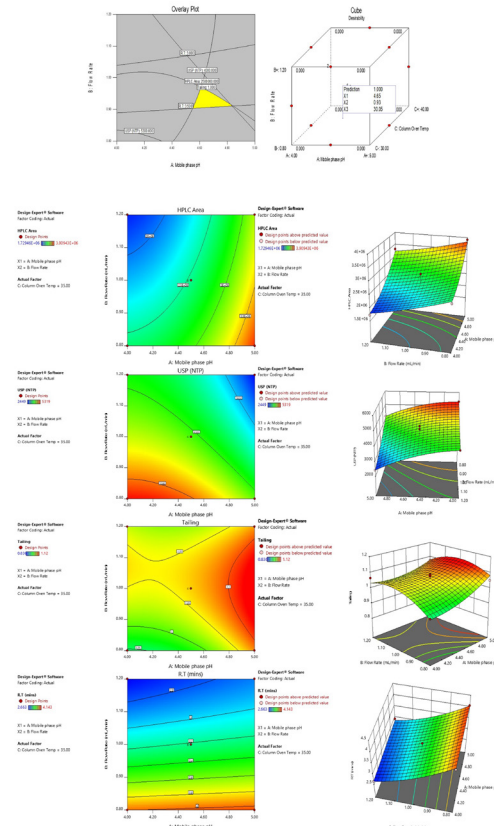


Figure 1: Desirability cube, overlay plot, 2D and 3D plot.

Table 2: ANOVA for response.

Source	Df	HPLC Area		USP (NTP)		Tailing		Retention time	
		F Value	p-value Prob > F	F Value	p-value Prob > F	F Value	p-value Prob > F	F Value	p-value Prob > F
Model	9	20.15	0.0001*	41.46	< 0.0001*	5.14	0.0154*	64.97	< 0.0001*
A-Mobile phase pH	1	126.18	< 0.0001*	144.15	< 0.0001*	10.10	0.0130*	5.25	0.0512
B-Flow Rate	1	35.90	0.0003*	204.96	< 0.0001*	2.19	0.1772	551.56	< 0.0001*
C-Column Oven Temp	1	0.81	0.3948	0.33	0.5795	1.00	0.3474	0.54	0.4837
AB	1	0.29	0.6069	0.95	0.3572	1.24	0.2974	0.60	0.4603
AC	1	0.31	0.5939	0.84	0.3870	0.026	0.8761	0.0004	0.9848
BC	1	2.78	0.1341	0.67	0.4377	14.27	0.0054*	2.39	0.1610
A ²	1	10.92	0.0108*	16.17	0.0038*	2.78	0.1338	0.0038	0.9522
B ²	1	0.52	0.4900	4.53	0.0660	9.40	0.0155*	24.23	0.0012*
C ²	1	4.63	0.0635	2.49	0.1532	5.30	0.0503	0.36	0.5625
Lack of Fit	3	0.85	0.5252	0.031	0.9919	3.58	0.1018	2.22	0.2036
R-Squared		0.9578		0.9790		0.8525		0.9865	
Adj R-Squared		0.9102		0.9554		0.6866		0.9713	
Pred R-Squared		0.7321		0.9642		-0.6775		0.8683	

Values of "Prob > F" less than 0.05 indicate significant model terms denoted by *.
Lack of fit is insignificant for all responses.

Equations

HPLC Area = 2542450 + 662623 * A -353449 * B + 53041.5 * C + 44671.8 * AB + 46318.5 * AC -139034 * BC + 263880 * A² + 57786.6 * B² -171940 * C².

USP (NTP) = 4097.5 -649.625 * A -774.625 * B -31.25 * C -74.75 * AB + 70 * AC -62.5 * BC -294.625 * A² + 155.875 * B² + 115.625 * C².

Tailing = 1.062 + 0.045375 * A + 0.021125 * B + 0.01425 * C -0.0225 * AB + 0.00325 * AC -0.07625 * BC + 0.03225 * A² -0.05925 * B² -0.0445 * C².

R.T. = 3.2165 + 0.061625 * A -0.631625 * B + 0.01975 * C + 0.0295 * AB -0.00075 * AC -0.05875 * BC -0.00225 * A² + 0.17925 * B² -0.022 * C².

Linearity

Method validation was done as per ICH Q2R1 after method development. Change in concentration showed up a linear relation with HPLC area when plotted. The R² value was found to be 1 with high correlation as given in Figure 2 and Table 3. The LOD of 0.08ppm and LOQ value of 0.25ppm show that the method is sensitive enough for lower concentrations.

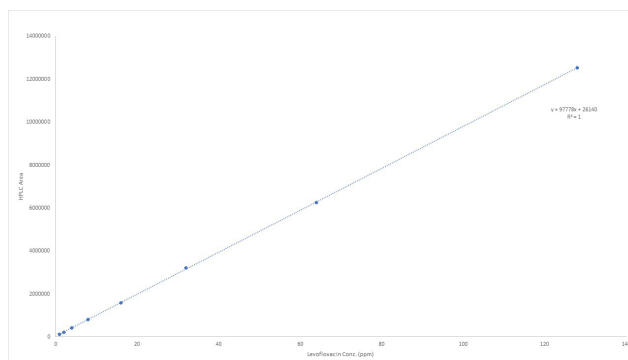


Figure 2: Linearity plot for levofloxacin.

System suitability

System suitability was run soon after establishing the linearity of the method at 64ppm. % RSD value was less than 0.5 with high theoretical plate count (>20000) and tailing factor less than 1.5 as given in Table 3 and exhibited in Figure 3.

Precision

Method precision and intermediate precision % RSD values lie between 0.2 and 0.6 which infer good precision of the method.

Table 3: Linearity, LOD and LOQ, System suitability, Precision.

LOD and LOQ		Linearity		System suitability (n=6 injections)		Method precision (n=6 injections)		Intermediate precision (n=6 injections)
Correlation	0.9992	Correlation	0.9999	Average	6188177	Average	6259080	6262012
R^2	0.9986	R^2	1	SD	±20056	SD	±17900	±36013.88
LOD	0.08 ppm			NTP	> 23000	Maximum	6276114	6299832
LOQ	0.25 ppm			Tailing factor	< 0.93	Minimum	6230032	6203917

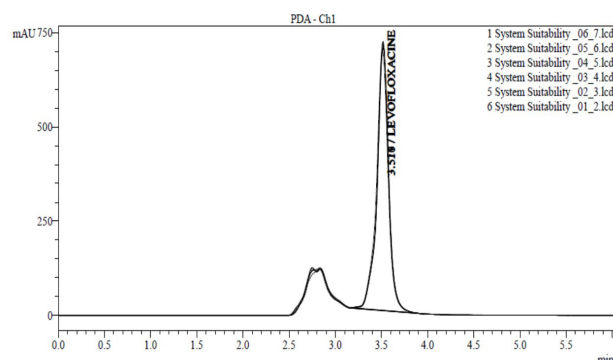


Figure 3: Chromatogram for system suitability.

Accuracy and Recovery

Accuracy and recovery studies were run at 3 levels. The percent recovery ranged from 99.8-101.6 with % RSD values <0.5. High recovery results indicate that the method can be used for quantitative quality control (QC) studies for various samples.

Robustness

The robustness study with deliberate variations in the chromatographic conditions had % RSD values <2, which prove the robustness of the developed method (Table 4).

Solution stability

Solution stability studies for sample were carried out at two conditions room temperature (25°C) and refrigeration (2-8°C) show that the sample prepared in diluent remained stable at both the conditions for 24 hrs as shown in Table 5.

Peak purity

Peak purity was established at 32 ppm concentration. Purity index was found to be 0.978 at a threshold value of 1, where purity index was less than threshold. So, we can conclude that the peak is pure as shown in Figure 4.

Application: Sample Analysis for Pharmacokinetics Study

Linear calibration curves were plotted for spiked plasma samples at a range of 0.5 ppm to 512 ppm. The concentration range was kept low to accommodate the range of expected plasma concentrations. The assay demonstrated good linearity in the selected range with a R^2 value nearing 0.999. The specificity studies for plasma samples were done after analysing overlaid chromatograms of spiked and blank samples for absence of interfering peaks at the RT of LH. Chromatograms of test, spiked and blank samples are given in Figure 5(A-C).

Application: Sample Analysis of Nano Formulations for LH Content Estimation

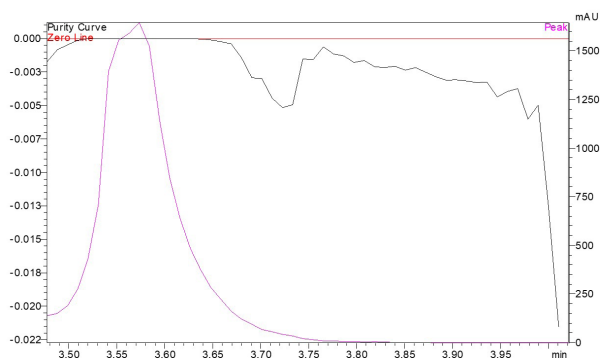
Nanoformulations containing LH in lipid and PLGA polymer poses problems of drug extraction by simple liquid-liquid extraction where diluents or organic solvents are utilised to extract the drug out of the polymeric shell. The prepared formulations being insoluble in water were difficult to characterise. Upon dissolution in acetonitrile or methanol, they gave an appearance of a homogenous solution to the eye, but during content measurements the drug contents remained much lower than expected. The problem was solved by using alkaline SDS solution to degrade the PLGA polymer chains and enable solubilisation of lipid. This process releases the LH in solution. The samples were further precipitated with diluent to extract out the free drug by centrifugation. The resultant solution so obtained is filtered and diluted to estimate drug content by HPLC. The presence of alkali and SDS in solution leads to change in pH during measurements which are controlled by making an acidic mobile phase which restricts movement of RT. The specificity of the method was analysed by running parallel samples of placebo, test and standard samples as given in Figure 5 (D-F). The absence of interfering peak at the RT of LH and no significant change in RT can be seen clearly in the representative chromatograms. Marketed tablet

Table 4: Accuracy and recovery and robustness.

Strength	Average area (n=4)	Amount recovered	% Recovery	Average	SD	%RSD
32 (50%)	3176431	32.21881	100.6838			
	3204916	32.51014	101.5942			
	3196614	32.42523	101.3288			
	3179636	32.25159	100.7862	3189399	13617.62	0.42
64 (100%)	6292802	64.09072	100.1417			
	6273758	63.89595	99.83742			
	6316568	64.33378	100.5215			
	6279722	63.95694	99.93272	6290713	18983.49	0.30
96 (150%)	9418814	96.06122	100.0638			
	9496665	96.85742	100.8931			
	9440183	96.27977	100.2914			
	9493824	96.82837	100.8629	9462372	38965.32	0.41
Robustness						
Condition	Average Area	Recovery	%RSD	RT	USP (NTP)	Tailing factor
Column oven temperature 25	6196729	98.60	1.29	3.557	5494.5	1.05
Column oven temperature 30	6229316	99.12	0.94	3.527	4855.5	0.96
Column oven temperature 35	6213473	98.87	0.11	3.526	4484	0.97
Flow rate 0.83	6357260	101.17	0.11	3.926	4424	0.91
Flow rate 0.93	6229316	99.12	0.94	3.527	4855.5	0.96
Flow rate 1.03	6179163	98.32	0.002	3.357	4658	0.95
Mobile phase composition 33% B	6261958	99.64	1.25	3.56	4545.5	1.20
Mobile phase composition 35% B	6229316	99.12	0.94	3.527	4855.5	0.96
Mobile phase composition 37% B	6172247	98.21	1.59	3.534	6789.5	0.94
Wavelength 293	6226636	99.08	0.93	3.527	4861	0.98
Wavelength 295	6229316	99.12	0.94	3.527	4855.5	0.96
Wavelength 297	6149412	97.85	0.94	3.527	4854.5	0.98

Table 5: Solution stability.

Time in hr	HPLC Area (n=3)	% Absolute difference
Initial	6411310	0
4 hr Room Temp	6411310	0
4 hr Refrigeration	6411250	0.000935846
12 hr Room Temp	6411598	0.004492062
12 hr Refrigeration	6411985	0.01052827
24 hr Room Temp	6411250	0.000935846
24 hr Refrigeration	6411480	0.002651564

**Figure 4: Peak purity profile.**

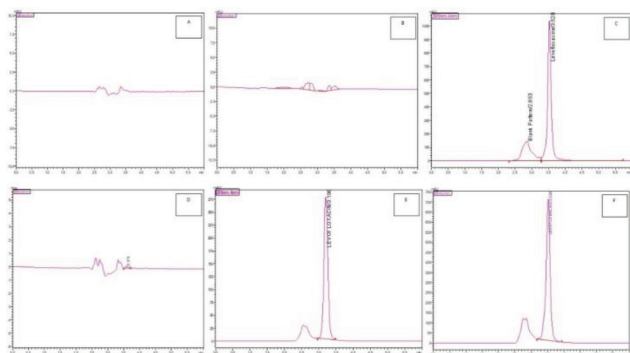


Figure 5: Specificity study A: Blank sample, B: Placebo plasma, C: Plasma sample, D: Placebo nanoformulation, E: Marketed, F: Nanoformulation.

Levaquin was used as a standard for LH estimation in nanoformulations. Standard I.P. assay procedure was used to prepare the samples.

CONCLUSION

The developed RP-HPLC method was quiet efficient in estimating LH content in nanoformulations, plasma samples and marketed conventional formulations with fast RT, resolution and sufficient specificity. The RP-HPLC method was entirely validated as per ICH Q2R1 guidelines. Since the method requires a short run time it can easily assess several samples in a short span of time. The method is robust, selective and simple with increased sensitivity for LH estimation. BBD used in development of method allowed better identification of significant factors affecting resolution. Until now there has been no novel method for estimation of LH in nanoformulations as well as in plasma samples for pharmacokinetic studies.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial or personal interests that can influence the work reported in this paper.

ABBREVIATIONS

ANOVA: Analysis of variance; **BBD:** Box Behnken Design; **BCS:** Biopharmaceutical Classification System;

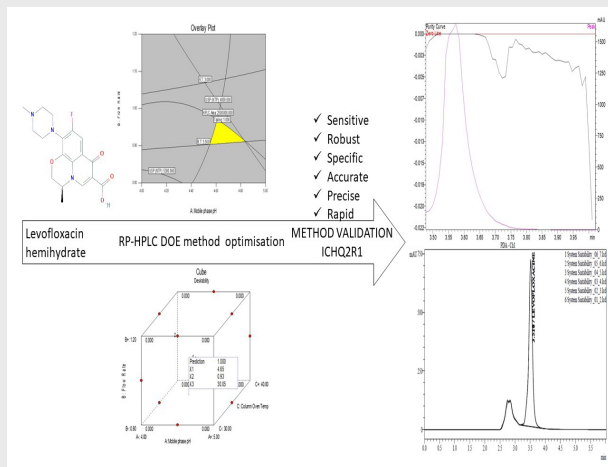
LH: Levofloxacin hemihydrate; **LOD:** Limit of detection; **LOQ:** Limit of quantification; **NTP:** Number of theoretical plates; **PLGA:** Poly D,L-lactic-co-glycolic acid; **PPM:** Parts per million; **QC:** Quality control; **RP-HPLC:** Reversed phase-High performance liquid chromatography; **RSD:** Relative standard deviation; **RT:** Retention time.

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PICTORIAL ABSTRACT



SUMMARY

A robust, rapid, simple, selective, sensitive RP-HPLC analytical method was developed and validated as per ICH Q2R1. Levofloxacin was extracted from plasma by liquid-liquid extraction and was measured using a validated RP-HPLC method with UV detector set at 295 nm. The DOE optimised developed method can be used for rapid estimation of levofloxacin in nanoformulations, marketed formulations and plasma samples without shift in RT. The significant reduction in retention time obtained by the developed method proves its efficiency in tapping the mobile phase costs as well as expediting the analytical process. Development of analytical method that uses minimal solvents with least preparation time also helps in reducing the errors.

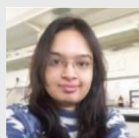
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