International Journal of Advances in Pharmaceutical Analysis
ISSN: 2277-9353 (Online)
Journal DOI: https://doi.org/10.7439/ijapa **Research Article**

RP-HPLC Method for Simultaneous Estimation of Levofloxacin Hemihydrate and Ornidazole from Tablet Formulation

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Abstract

An accurate, simple, reproducible and precise reverse phase high-performance liquid chromatographic method for the determination of levofloxacin hemihydrate and ornidazole has been developed and validated. The separation of levofloxacin, ornidazole and fluconazole (internal standard) was achieved on a Luna C_{18} (5µ, 250 mm X 4.60 mm i.d.) column using UV detection at 254 nm. The mobile phase was consisted of 0.5 % triethylamine in water and acetonitrile (65: 35) pH adjusted to 3.0 with 5 % phosphoric acid. The flow rate was 0.8 ml/min. The retention time of levofloxacin hemihydrate and ornidazole were 3.17 min and 6.63 min respectively. The linearity of levofloxacin and ornidazole were found to be 4 – 24 mcg/ml (r^2= 0.9991) and 8 – 48 mcg/ml (r^2= 0.9992), respectively. The method has been successively applied to pharmaceutical formulation. No chromatographic interferences from the tablet excipients were found. The method has been shown to be linear reproducible, specific and rugged.

Keywords: Levofloxacin hemihydrate, Method validation, Ornidazole, Reversed phase HPLC.

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***Article History:**
Received: 24/07/2026
Revised: 17/08/2026
Accepted: 28/08/2026
DOI: https://doi.org/10.7439/ijapa.v16i1.5937

How to cite: Khan E, Mandawar K. and Jain P. RP-HPLC Method for Simultaneous Estimation of Levofloxacin Hemihydrate and Ornidazole from Tablet Formulation. *International Journal of Advances in Pharmaceutical Analysis* 2026; 16(1): e5937. Doi: 10.7439/ijapa.v16i1.5937 Available from: https://ssjournals.co.in/index.php/ijapa/article/view/5937

1. Introduction

Levofloxacin (LEVH), chemically (-)-(S)-9-fluro-2, 3-dihydro- 3methyl-10- (4-methyl-1-piperzinyl)-7-oxo-7H pyrido [1, 2, 3-de]-1, 4- benzoxazine-6-carboxylic acid hemihydrate is synthetic fluroquinolone anti-infective agent. It is optically active isomer of ofloxacin. It is act by inhibiting bacterial DNA gyrase enzyme which is required for DNA replication and thus cause bacterial lysis. Levofloxacin is used to treat bacterial conjunctivitis, sinusitis, chronic bronchitis, community-acquired pneumonia caused by penicillin resistant strains of streptococcus pneumonia, skin and skin structure infections complicated urinary tract infections and acute pyelonephritis [1-3]. Ornidazole(ORN), chemically [1-chloro-3-(2-methyl-5-nitroimidazole -1-yl) 2-propanol] a 5-nitroimidazole derivative is a broad spectrum of protozoal and antibacterial activity. It shows antibacterial action against all anaerobic cocci, anaerobic gram-ve bacilli including bacterides species and anaerobic spore forming gram^{+ve} bacilli. In anaerobic microorganism ornidazole is converted to active form by reduction of its nitro group; this gets bound to DNA and prevents nucleic acid formation. It is very effective in infections due to entomoeba histolytica, giardialambia and trichomoniasis.

Many spectrophotometric methods, HPLC and HPTLC [4-19] have been reported for the determination of LEVH [4-10] and ORN [13-19] in single or combined pharmaceutical dosage form or biological fluids. But none of these methods demonstrate the simultaneous estimation of these two drugs in combination in pharmaceutical dosage form. The method are simple, reduce the duration of the analysis and suitable for routine determination of two drugs.

2. Experimental

2.1 Chemicals and Reagents

Levofloxacin and ornidazole were obtained from Glenmark pharmaceuticals, Nashik, (India). Fluconazole (Internal standard) was obtained from Torrent Pharmaceuticals, Ahemdabad, (Gujrat). Acetonitrile, Triethylamine, Double distilled water, phosphoric acid used were of HPLC grade obtained from Qualigens Fine Chemicals (Mumbai, INDIA).

2.2 Instrument

All HPLC measurments were made on a Shimadzu corporation system (Analytical Instruments division, Kyoto, Japan) consisting pump LC-10 ATvp equipped with universal injector (Rheodyne) with injection volume of 20 µl, ultra-visible (UV-VIS) detector SPD-10 ATvp Shimadzu class M-10 software. The separation was performed on a Luna C_{18} column (5 µ, 250 X 4.6 mm i.d.) was used. A mixture 0.5% triethylamine in water-acetonitrile (65:35, v/v) pH adjusted 3.0 with 5% phosphoric acid, was used as a mobile phase at a rate of 0.8 ml/min. The mobile phase was filtered through 0.45 µ Millipore membrane filter and degassed. Detection was done at 254 nm. The separation was carried out at room temp.

2.3 Preparations of solutions

2.3.1 Standard solutions and calibration graphs

2.3.1.1 Determination of λ_{max}

From the stock solutions, 1.0 mL of LEV and 2.0 mL of ORN was transferred to two separate 10 mL volumetric flasks and the volume was adjusted to the mark with water i.e. strength obtained was 10 µg/mL for LEV and 20 µg/mL of ORN. Both the drug solutions were scanned separately between 200 nm to 400 nm. The overlain spectrum of both drugs was recorded (Shown in **Fig.1.**) and two wavelengths 289.0 nm (λ max of LEV) nm and 320.0 nm (λ max of ORN) were selected for estimation of drugs using simultaneous equation method.

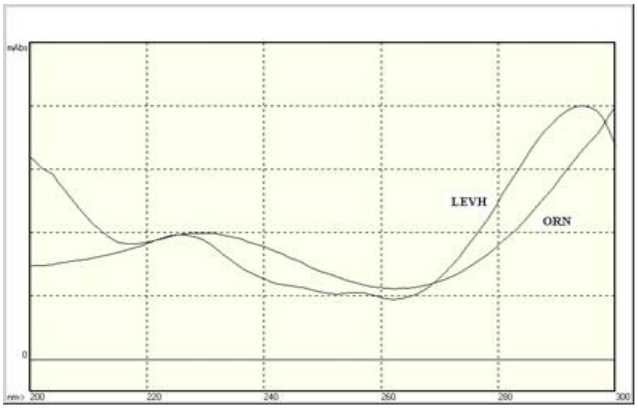

Fig.1. Overlay spectra of levofloxacin hemihydrate and ornidazole

Standard mixed stock solution was prepared by dissolving 10 mg of levofloxacin hemihydrate and 20 mg of ornidazole in 100 ml mobile phase (I). Fluconazole (IS) stock solution was made at a final concentration 100 mcg/ml using mobile phase (II). Aliquot of standard stock solution I, II in six different 10 ml volumetric flask and diluted up to the mark with mobile phase such that the final concentration of LEVH and ORN were in the range 4 – 24 mcg/ml, 8 – 48 mcg/ml with 50 mcg/ml FLU. Triplicate 20 µl injections were made for each standard solution to see the reproducibility of the detector response at each concentration level.

The peak area ratio of standard to internal standard was plotted against the concentration of the drug to obtain the calibration graph. The results were subjected to regression analysis to calculate calibration equation and correlation coefficients. Atypical chromatogram obtained was shown in **fig.2.**

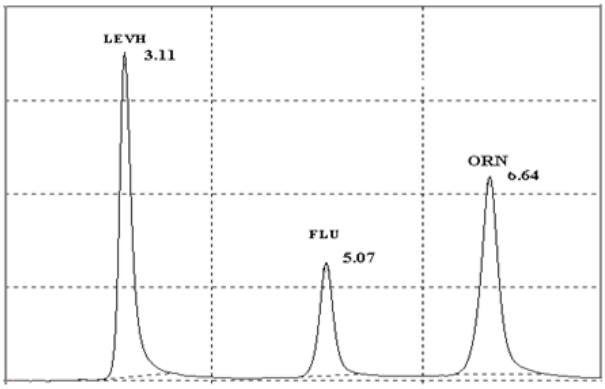

Fig. 2. Chromatogram of standard LEV (8mcg.ml): (t_R : 3.11±0.05), ORN (16mcg/ml): (t_R : 6.64±0.04) and FLU(internal standard 50 mcg.ml): (t_R : 5.07±0.03), measured at 254 nm, mobile phase 0.5 % triethylamine in water:acetonitrile(65:35) pH adjusted to 3.2 with 5 % phosphoric acid.

2.3.2 Sample Solution

To determine the content of levofloxacin hemihydrate and ornidazole simultaneously in conventional tablets (Label claim 250 mg levofloxacin hemihydrate and 500 mg ornidazole per tablet, combination tablet containing both analytes); the twenty tablets were weighed, their mean weight determined and they were finely powered and powder equivalent 25 mg levofloxacin hemihydrate and ornidazole was transferred into a 100 mL volumetric flask containing 50 mL mobile phase, sonicated for 30 min. and diluted to 100mL with mobile phase. The resulting solution was filtered, using 0.45 µm filter (Millifilter, Milford, MA). The solution was further diluted to get concentration 8 mcg/ml LEVH and 16 mcg/ml of ORN with 50 mcg/ml of

standard FLU solution was injected into HPLC, six times, under the conditions described above. The peak area ratio of sample to internal standard were measured at 254 nm for LEVH and ORN, respectively and their concentrations in the sample were determined using multilevel calibration developed on the same HPLC system under the same conditions using linear regression equation.

3. Method validation and results

3.1 Optimization of mobile phase

The mobile phase was chosen after several trials with acetonitrile, water and triethylamine in various proportions and at different P^H values. A mobile phase consisted of 0.5 % triethylamine in water: acetonitrile (65:35, v/v) pH adjusted to 3.0 with 5 % phosphoric acid was selected to achieve maximum separation and sensitivity. The effects of flow rates in the ranges of 0/5 to 1.5 ml/min were examined. A flow rate of 0.8ml/min gave reasonable separation time, using reverse phase C_{18} column, the retention times of LEVH, ORN and FLU (I.S.) were observed 3.17, 6.63 and 5.07 min, respectively. The total time of analysis was less than 10 min. The solution containing LEVH, ORN and FLU exhibited absorption at 254 nm and hence this wavelength was chosen for the analysis.

3.2 Linearity and Calibration

The calibration curves were linear in the studied range. The calibration curve equation is y = bx + c, where y represents the ratio of LEVH/ORN peak area to FLU peak area and x represents the ratio of LEVH/ORN concentrations to that of FLU. The mean equation of the calibration curve (n = 3) obtained was y = 0.17774x + 1.642 for LEVH and y = 0.1195x + 1.014 for ORN. Excellent linearity was obtained for both drugs between peak area ratios and concentrations of 4 – 24 mcg/ml with $r^2 = 0.9991$ and 4 – 48 mcg/ml with $r^2 = 0.9992$ for LEVH and ORN, respectively.

3.3 Precision

Accuracy of the assay method was determined for both intra-day and interday variations using the six analysis of the samples. Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability refers to the use of the analytical procedure within a laboratory even a short period of time that was evaluated by assaying the samples during the same day. Intermediate precision was assessed by comparing the assays in different days (3days) and expressed as %RSD. The intra-day and inter-day precision has been depicted in **Table I.**

Table I: Precision

Component	Inter-day precision		Intra-day precision	
	%RSD	SE	%RSD	SE
LEV [a]	0.97	0.31	1.44	0.090
ORN [b]	0.61	0.019	0.57	0.037

a = Average of six concentrations of 8 mcg/ml of LEV
b = Average of six concentrations of 16 mcg/ml of ORN

3.4 Recovery

To ensure the reliability and accuracy of the method recovery studies were carried out by mixing a known quantity of standard drug with pre analysed sample and contents were re analysed by the proposed method. Recovery studies was carried out by applying the method to drug sample to which known amount of LEVH and ORN corresponding to 80, 100 and 120 % had been added (Standard addition method). At each level of the amount three determinations were performed and the results obtained were compared with excepted results. The method used for extraction and subsequent estimation of LEVH and ORN from pharmaceutical dosage form after spiking with additional drug afforded recovery of 98 – 102 % and mean recovery for LEVH and ORN from the marketed formulation listed in Table II.

Table II: Recovery

Excess drug added to the analyte (%)	Theoretical content (mcg/ml)	Recovery %	%RSD	*SE*
LEVH				
0	8	100.11	0.54	0.31
80	6.4	100.94	0.77	0.44
100	8	99.96	1.74	1.00
120	9.6	99.84	1.82	1.05
ORN				
0	16	100.42	0.92	0.53
80	12.8	100.73	0.91	0.52
100	16	100.39	0.51	0.29
120	19.2	100.08	1.46	0.84

3.5 Sensitivity

The sensitivity of measurements of LEVH and ORN by the use of the proposed method was estimated in terms of the Limit of Quantitation (LOQ) and the lowest concentration detected under the chromatographic conditions as the Limit of Detection (LOD).

The LOQ and LOD were calculated by the use equation LOQ = 10 x N/B and LOD = 3.3 x N/B, where 'N' is standard deviation of the peak areas of the drugs (n=6), taken as a measure of noise, and 'B' is the slope of the corresponding calibration curve.

The Limit of Quantitation (LOQ) was calculated to be 2.48 mcg/ml and 4.71mcg/ml and the limit of detection was calculated to be 0.81mcg/ml and 1.55 mcg/ml for LEVH and ORN, respectively.

3.6 Ruggedness

The ruggedness of the HPLC method was evaluated by carrying out the analysis of the sample solution, the same chromatographic system and the same column on different analyst. The ruggedness of the proposed method was evaluated by performing the determinations by two different analysts, the assay results (n=6) were found to give 99.45 %, 99.76 % of levofloxacin and 99.65 %, 99.47 % of ornidazole.

3.7 System Suitability Tests

The System Suitability Tests of the HPLC method was determined by the complete separation of LEVH and ORN along with other parameter like retention time (t_r), capacity factor (k), tailing /asymmetrical factor (t) etc. The specificity of the HPLC method is illustrated in FIg.7 where complete separation of LEVH and ORN was noticed in presence of tablet excipients. The average retention time of LEVH and ORN were found to be 3.58±0.02 and 6.58±0.03 min, respectively for six replicates. The peaks obtained were sharp and have clear baseline separation.

Parameters that were studied to evaluate the suitability of the system were

a) Number of theoretical plates.
b) Tailing factors/ asymmetric factors.
c) Resolution.
d) Retention time

The values of system suitability tests were shown in **Table III.**

Table III: System Suitability Parameters

S. N.	Parameter	LEVH	ORN
1	Capacity factor	0.60	1.09
2	Theoretical plates	3224	7138
3	Tailing factor	1.57	1.11
4	Resolution factor	3.56	5.41
5	Retention time in minutes	3.11	6.64

3.8 Stability studies

Two different concentrations of LEVH (8 and 12 mcg/ml) and ORN (16 and 24 mcg/ml) were prepared from sample solution and stored at room temp for 3 hours, 24 hours and 48 hours. They were then injected into the HPLC system and no additional peak was found in the chromatogram indicating the stability of LEVH and ORN in the sample solution. The results are listed in **Table IV.**

Table IV. : Results of Stability studies:

Drug	% Drug loss ± SD		
	3 Hours	24 Hours	48 Hours
LEVH	99.46± 1.56	96.03 ± 0.85	90.04 ± 0.11
ORN	99.63 ± 1.68	95.66 ± 1.30	91.63 ± 0.26

3.9 Analysis of marketed formulation

The peaks at t_r 3.58 (LEVH) and 6.58 min (ORN) were observed in the chromatogram of the drug samples extracted from tablets. Experimental results of the amount of LEVH and ORN in tablets, expressed as percentage of label claim were in good agreement with the label claim were in good agreement, thereby suggesting that there is no interference from any excipients. Low values of RSD indicated high precision of the proposed method. The results were shown in **Table V.** The data of summary of validation parameter are given in **Table VI.**

Table V.: Assay of Levofloxacin and Ornidazole:

LEVH/ORN tablet (mg/tablet)	LEVH/ORN found (mg/tablet)^n	%RSD	SE
Levofloxacin (250)	250.28	0.54	0.55
Ornidazole (500)	502.13	0.92	1.89

n=6

Table VI. Summary of validation parameter

Parameter	LEVH	ORN
Linearity range	4 – 24 mcg/ml	8 – 48 mcg/ml
Correlation coefficient	0.9991	0.9992
Limit of detection	0.81	1.55
Limit of quantitation	2.48	4.71
Recovery (n=3)	100.21±0.96	100.40±0.95
Precision(%RSD)		
Intra-day(n=3)	1.44	0.57
Inter day(n=3)	0.97	0.61

4. Discussions

A rapid, specific, isocratic HPLC method has been developed for the determination of LEVH and ORN in pharmaceutical dosage form. The data demonstrate that the analytical method we have developed showed acceptable linearity, precision and accuracy over the concentration range. The method uses a simple mobile phase composition, easy to prepare with little or no variation. The rapid run time of 10 min and the relatively low flow rate (0.8 ml/min) allows the analysis of large no. of samples with less mobile phase that proves to be cost effective. As per the current regulatory requirements resolution between the two components should more than 3.0, tailing factor should be less than 2 and theoretical plates should be more than 2000. It is evident from table 4 that method developed for these two drugs in combination is passing the standards of regulatory requirements. The method was found to be precise and accurate with low values of relative standard

deviation. High % recovery values show that the compounds are completely extracted from pharmaceutical dosage form. The proposed RP-HPLC method is accurate, simple, rapid and selective for the simultaneous estimation of LEVH and ORN in tablet dosage form by internal standard method hence it can be conviently adopted for the routine quality control analysis of the tablet dosage form.

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