# Validated HPTLC Method for Simultaneous Estimation of Levofloxacin Hemihydrate and Ornidazole in Pharmaceutical Dosage Form

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### Abstract

A simple, rapid, and accurate high-performance thin-layer chromatography (HPTLC) method is described for the simultaneous determination of levofloxacin hemihydrate and ornidazole in tablet dosage form. The method is based on the HPTLC separation of the two drugs followed by densitometric measurements of their spots at 298 nm. The separation is carried out on Merck TLC aluminium sheets of silica gel 60 $F_{254}$ using *n*-butanol–methanol–ammonia (5:1:1.5, v/v/v) as mobile phase. The linearity is found to be in the range of 50–250 and 100–500 ng/spot for levofloxacin hemihydrate and ornidazole, respectively. The method is successively applied to pharmaceutical formulation because no chromatographic interferences from the tablet excipients are found. The suitability of this HPTLC method for the quantitative determination of the compounds is proved by validation in accordance with the requirements laid down by International Conference on Harmonization (ICH) guidelines.

## Introduction

Levofloxacin (LEVH), chemically (–)-(S)-9-fluro-2, 3-dihydro-3-methyl-10-(4-methyl-1-piperzinyl)-7-oxo-7H pyrido [1,2,3-de]-1,4-benzoxazine-6-carboxylic acid hemihydrate, is synthetic fluroquinolone. It is a third generation bactericidal quinoline with improved gram+ and gram– enteric activity (pseudomonas, mycoplasma, legionella, etc.) that binds DNA–DNA gyrase (topoisomerase II) complex and blocks further DNA replication; it also blocks topoisomerase IV interferes with the separation of interlocked replicated DNA molecules. 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 by 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, has a broad spectrum of protozoal and antibacterial activity. It shows antibacterial action against all anaerobic cocci and anaerobic gram-ve bacilli (*Porphyronomas, Fusobacterium*) including protozoa viz. *E. histolytica, T. vaginalis, G. intestinalis,* etc. as well as anaerobic spore forming gram+ve bacilli (*Peptostreptococcus, Clostridium, B. fragilis, Prevotella*). In an anaerobic microorganism, ornidazole is converted to its active form by reduction of its nitro group, this gets bound to DNA and precents nucleic acid formation (11,12).

Many spectrophotometric methods, high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) (4–19), have been reported for the determination of levofloxacin (4–10) and ornidazole (13–19) in single or combined pharmaceutical dosage forms 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 suitble for routine determination of two drugs.

## Experimental

### Materials and reagents

Levofloxacin hemihydrate and ornidazole were kindly supplied as a gift sample by Glenmark Pharmaceuticals, Ltd., (Nashik, India). Methanol, ammonia, and *n*-butanol were used as solvents to prepare the mobile phase. All the reagents used were of analytical-reagent grade (S.D. Fine. Chemicals, Mumbai, India) and used without further purification.

### Instrumentation and chromatographic conditions

The samples were spotted in 6-mm wide bands with a Camag 100-µL sample (Hamilton, Bonaduz, Switzerland) syringe on precoated silica gel aluminium plate 60 F-254 (10 × 10) with 250-µm thickness (E. Merck, Darmstadt, Germany), supplied by Anchrom technologists, (Mumbai) using a Camag Linomat

V (Camag, Geneva, Switzerland). The plates were prewashed with methanol and activated at 110°C for 5 min prior to chromatography. A constant application rate of 0.1 µL/spot was employed, and the space between two bands was 5 mm. The slit dimension was kept at 6 mm × 0.45 mm and 10 mm/spot scanning speed was employed. The monochromator band width was set at 20 nm and 320 cut off filter; each track was scanned thrice, and base line correction was used. The mobile phase consist of $n$-butanol–methanol–ammonia (5:1:1.5 v/v/v). Linear ascending development was carried out in a 10 × 10-cm twin trough glass chamber (Camag, Muttenz, Switzerland) (dimensions: length × width × height = 12 cm × 4.7 cm × 12.5 cm). The chamber saturation time for mobile phase was 20 min at room temperature (25°C ± 2) and at a relative humidity of 60% ± 5. The length of chromatogram run was 8 cm and approximately 40 min, subsequent to the development. TLC plates were dried in a current of air with the help of an air drier. Densitometric scanning was performed on Camag TLC scanner III in the reflectance–absorbance mode at 298 nm for all measurements and operated by WINCATS software version 1.3.0. The source of radiation utilized was a deuterium lamp, which continuously emits a UV spectrum between 200 nm to 400 nm. Evaluation was via peak area with linear regression.

### Standard solutions and calibration graphs

A Combined standard stock solution containing 100 µg/mL levofloxacin hemihydrate and 200 µg/mL ornidazole was prepared in methanol. Calibration was done by applying mixed standard solution ranging from 0.5–2.5 µL by a micro liter syringe with the help of automatic sample applicator Linomat V on TLC plate, which gives a concentration of 50–250 ng/spot of levofloxacin hemihydrate and 100–500 ng/spot of ornidazole. Each concentration was spotted six times on the TLC plates. The plate was developed on a previously described mobile phase. The peak areas plotted against the corresponding concentrations to obtain the calibration graphs.

### Sample preparation

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), twenty tablets were weighed, their mean weight determined, and finely powered. The powder equivalent to 10 mg levofloxacin hemihydrate and 20 mg ornidazole was transferred into a 100-mL volumetric flask containing 50 mL methanol, sonicated for 30 min, and then diluted to mark with the same solvent. The resulting solution was filtered using 0.45-µm filter (Millipore, Milford, MA). The solution (1 µL, containing 100 ng of LEV and 200 ng of ORN) was spotted for assay of levofloxacin hemihydrate and ornidazole.

## Method Validation (20–29)

### Optimization of HPTLC method

Initially $n$-butanol and methanol in the ratio of 3:3 (v/v) was tried for both drugs simultaneously. The spots were not developed properly, and dragging was observed. Then, methanol and $n$-butanol in the ratio of 5:2 (v/v) was tried. The developed spots were diffused. To the described mobile phase, 0.5 mL ammonia was added. Both the peaks were symmetrical in nature, and tailing was observed. To improve the resolution, the volume of ammonia was increased by 1 mL, and the value of methanol was reduced by 1 mL. Ultimately, mobile phase consisting of $n$-butanol–methanol–ammonia (5:1:1.5, v/v/v) gave good resolution. Both the peaks were symmetrical in nature, and no tailing was observed when plates were scanned at 298 nm. Well-defined spots were obtained when the plate was activated at 110°C for 5 min, and the chamber was saturated with the mobile phase for 20 min at room temp (Figure 1).

### Linearity

Mix standard solutions equivalent to 50, 100, 150, 200, and 250 ng/µL of levofloxacin hemihydrate and 100, 200, 300, 400, and 500 ng/µL of ornidazole were spotted on the prewashed TLC plates. The plates were developed, dried, and scanned as described earlier. The calibration curves were constructed by plotting peak areas against the corresponding concentrations of both the drugs (ng/spot) individually. Levofloxacin hemihydrate and ornidazole showed good correlation coefficient in the concentration range of 50–250 ng/spot ($r = 0.9998$) and 100–500 ng/spot ($r = 0.999$) over the concentration range

### Precision and reproducibility of run time

Precision studies were performed by using a standard solution containing both the drugs with the concentrations of drugs covering the entire calibration range. The precision of the method in terms of intra-day variation (%RSD) was determined by analyzing standard drug solutions within the calibration range, three times on the same day. Inter-day precision (%RSD) was assessed by analyzing the standard drug solutions within the calibration range on three different days over
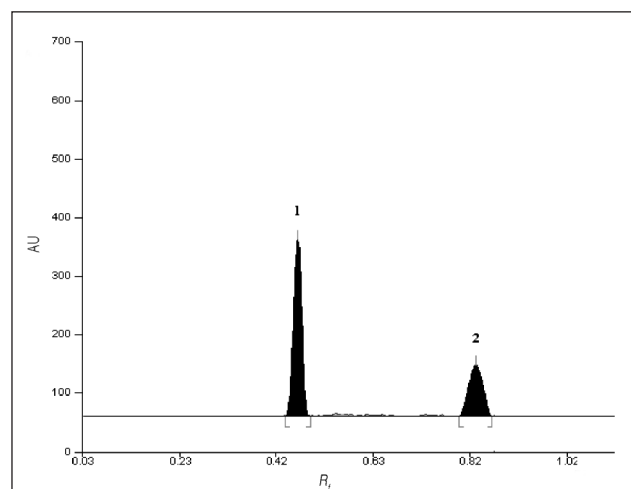


**Figure 1.** Densitogram of standard levofloxacin hemihydrate (100 ng/spot), Peak 1 ($R_f$ 0.42 ± 0.02) and ornidaole (200 ng/spot), Peak 2, in a ratio of 1:2 measured at 290 nm, with a mobile phase of n-butano–methanol–ammonia (5:1:1.5, v/v/v). (Typical HPTLC chromatogram of levofloxacin and ornidaole).

a period of one week. For reproducibility, blank plates and spotted plates were run under identical conditions at constant run length of 9 cm. The %RSD values given in Tables I and II show that the proposed method provides acceptable reproducibility in terms of run time and intra- and inter-day variation for levofloxacin and ornidazole.

### Recovery

Recovery studies were carried out by applying the method to a drug sample to which known amount of levofloxacin hemihydrate and ornidazole corresponding to 80%, 100%, 120% of the standard drug solutions were spiked, and the percentage recoveries were found to be within the limits. Sample stock solution of tablet formulation of 100 ng/µL of levofloxacin hemihydrate and 200 ng/µL of ornidazole was prepared. To the above prepared solutions, 80%, 100%, and 120% of the standard drug solutions were spiked, and the percentage recoveries were found to be within the limits as given in Table III.

### Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The mobile phase resolved both the drugs very efficiently, as shown in Figure 1. The spot for the levofloxacin hemihydrate and ornidazole sample was confirmed comparing the retention factor ($R_f$) and spectra of the spot with that of standard shown in Figures 2 and 3. The wavelength 298 ($\lambda_{max}$ of levofloxacin hemihydrate) for detection peak purity of levofloxacin hemihydrate and ornidazole was assessed by comparing the spectra at three different levels [i.e., peak start (S), peak apex (M), and peak end (E) positions of the spot].

### Sensitivity

The sensitivity of measurements of levofloxacin hemihydrate and ornidazole 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).

LOQ and LOD were calculated by the use of the equation LOD = 3.3 × N/B and LOQ = 10 × 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. For levofloxacin hemihydrate, LOD and LOQ was found to be 17.3 ng and 5.7 ng. And, for ornidazole, LOQ and LOD was found to be 28.3 and 0.9 ng.

### Robustness and Ruggedness

Robustness is a measure of the capacity of a method to remain unaffected by small but deliberate variations in the method conditions and is an indication of the reliability of the method. Robustness was assessed by altering the migration distance of the solvent front. The assay result has been given in Table IV. The amount of mobile phase, temperature, and relative humidity was varied in the range of ± 5%. The plates were pre-washed by methanol and activated at 60°C ± 5°C for 2, 5, and 7 min, respectively, prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20, 40, and 60 min (Table V). 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% and 99.76% of levofloxacin and 99.65% and 99.47% of ornidazole.

### Repeatability

Repeatability of sample application was assessed by spotting

**Table I. Reproducibility of Run Time***

| Plate condition[†] | Run time (min)[‡] | SD | %RSD |
|---|---|---|---|
| Blank plate | 42.30 | 0.61 | 1.44 |
| Plate spotted with standards | 42.16 | 0.63 | 1.49 |

* n = 6
† Plates pre-treated with methanol and activated at 110°C.
‡ Development was performed in the ascending direction at constant run length of 9 cm.

**Table II. Intra and Interday Precision of Levofloxacin Hemihydrate and Ornidazole**

| Drug | Concentration (ng/spot) | Intraday precision (%RSD, n = 3) | Interday precision (%RSD, n = 3) |
|---|---|---|---|
| Levofloxacin hemihydrate | 100 | 0.028 | 0.045 |
| | 150 | 0.021 | 0.045 |
| | 200 | 0.014 | 0.02 |
| Ornidazole | 200 | 0.146 | 0.19 |
| | 300 | 0.079 | 0.15 |
| | 400 | 0.11 | 0.083 |

**Table III. Recovery Studies**

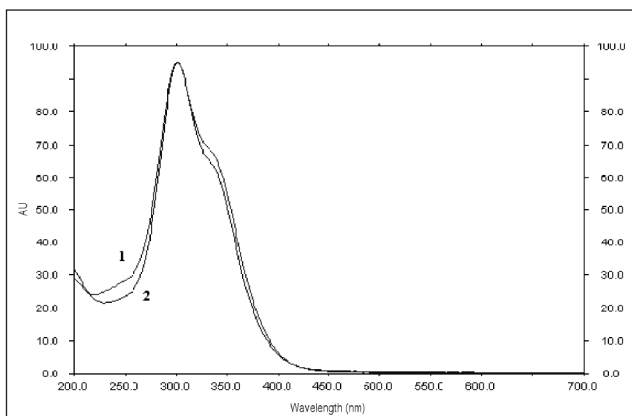| Component | Label claim (mg tablet) | Initial amount (ng) | Amount added (%) | Amount recovered (ng) | %Recovered | %RSD |
|---|---|---|---|---|---|---|
| Levofloxacin hemihydrate | 250 | 100 | 0 | 99.45 | 99.45 | 0.24 |
| | | 100 | 80 | 179.73 | 99.85 | 0.10 |
| | | 100 | 100 | 199.64 | 99.82 | 0.095 |
| | | 100 | 120 | 220.13 | 100.06 | 0.019 |
| Ornidazole | 500 | 200 | 0 | 199.2 | 99.60 | 0.28 |
| | | 200 | 80 | 358.02 | 99.45 | 0.20 |
| | | 200 | 100 | 402.08 | 100.52 | 0.054 |
| | | 200 | 120 | 441.18 | 100.27 | 0.20 |

**Figure 2.** Peak purity spectra of standard 1 levofloxacin, sample 2 extracted from a levofloxacin hemihydrate–ornidazol tablet, scanned at the peak start, peak apex, and peak-end positions of the spot (correlation > 99).
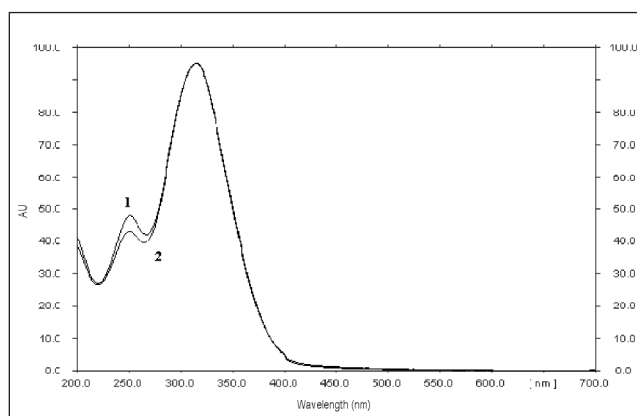


**Figure 3.** Peak purity spectra of standards 1ornidazole, sample 2 extracted from a levofloxacin hemihydrate–ornidazole tablet, scanned at the peak-start, peak apex, and peak-end positions of the spot (correlation > 0.99).

### Table IV. Results of Robustness Studies

| Development distance | Levofloxacin Assay (%) | Ornidazole Assay (%) |
|---|---|---|
| 7.0 | 99.56 | 99.24 |
| 7.5 | 99.15 | 98.87 |
| 8.0 | 99.67 | 99.39 |

### Table V. Results of Robustness Studies

| Parameter | SD of peak area* | | %RSD | |
|---|---|---|---|---|
| | Levofloxacin | Ornidazole | Levofloxacin | Ornidazole |
| Temperature | 1.23 | 1.07 | 1.03 | 0.94 |
| Relative humidity | 1.65 | 1.71 | 1.35 | 1.29 |
| Plate pretreatment | 0.72 | 0.59 | 0.52 | 0.38 |
| Time from spotting to chromatography | 0.52 | 0.49 | 0.43 | 0.35 |
| Time from chromatography to spotting | 0.46 | 0.43 | 0.29 | 0.31 |

\* $n = 5$.

### Table VI. Results of Stability Studies:

| Drug | % Drug loss | ± SD [%] | |
|---|---|---|---|
| | 3 H | 24 H | 48 H |
| Levofloxacin hemihydrate | No Loss | 3.97 ± 0.085 | 9.96 ± 0.11 |
| Ornidazole | No Loss | 4.34 ± 0.30 | 8.37 ± 0.26 |

(100 ng/µL) of drug solution 7 times on a TLC plate followed by development of plate and recording the peak area for 7 spots. The %RSD for peak area values of levofloxacin hemihydrate and ornidazole was found to be 0.48 and 0.12, respectively.

Repeatability of measurement of peak height and area were determined by spotting 10 µL of standard drug solution on TLC plate and developing the plate. The separated spot was scanned 7 times without changing the positions of the plate and % RSD for measurement of peak area of levofloxacin hemihydrate and ornidazole were 0.26 and 0.13, respectively.

**System suitability**

According to USP 28, system suitability tests are an integral part of a chromatographic analysis and should be used to verify that the resolution and reproducibility of the chromatographic system is adequate for the analysis. To ascertain, the effectiveness of the method developed in this study, system suitability tests were performed on freshly prepared standard stock solutions of levofloxacin and ornidazole.

**Stability studies**

To test the stability of drugs on the TLC plates, the concentration of applied drugs were determined by scanning the plate within 3, 24, and 48 h, after development. The results of the stability studies are given in Table VI.

**Analysis of the marketed formulation**

The spots at $R_f$ 0.42 (for levofloxacin) and 0.84 (for ornidazole) were observed in the densitogram extracted from tablets. There were no interferences from the excipients commonly present in the tablets. Results are given in the Table VII. The low %RSD value indicates the suitability of this method for

**Table VII. Assay of Levofloxacin and Ornidazole**

| Component | Label claim (mg) | Amount found (mg) | Amount found (%) | % RSD | SEM |
|---|---|---|---|---|---|
| *Levofloxacin* | 250 | 249.02 | 99.61 | | |
| | 250 | 248.77 | 99.51 | | |
| | 250 | 248.12 | 99.25 | 0.18 | 0.10 |
| | Mean | 248.63 ± 0.46 | | | |
| *Ornidazole* | 500 | 496.5 | 496.5 | | |
| | 500 | 491.1 | 491.1 | | |
| | 500 | 493.9 | 493.9 | 0.54 | 0.02 |
| | Mean | 493.83 ± 2.70 | | | |

**Table VIII. Summary of Validation Parameter**

| Parameter | HPTLC-Densitometric | |
|---|---|---|
| | Levofloxacin | Ornidazole |
| Linearity range | 50–250 ng/spot | 100–500 ng/spot |
| Correlation coefficient | 0.9998 | 0.9996 |
| Limit of detection | 0.11 | 0.45 |
| Limit of quantitation | 0.36 | 2.91 |
| Recovery ($n = 3$) | 99.91 | 100.08 |
| Precision (%RSD) | | |
| Repeatability | 0.03 | 0.14 |
| Inter-day ($n = 3$) | 0.036 | 0.141 |
| Intra-day ($n = 3$) | 0.021 | 0.11 |
| Robustness | 99.46 | 99.16 |
| Specficity | 0.997 | 0.996 |

routine analysis of levofloxacin hemihydrate and ornidazole in pharmaceutical formulation. The data of summary of validation parameter are given in Table VIII.

## Conclusion

The proposed HPTLC method provides simple, accurate, and reproducible quantitive analysis for simultaneous determination of levofloxacin and ornidazole in tablets. The method was validated as per ICH guidelines.

## Acknowledgments

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