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Assay of Levofloxacin and Ornidazole in Combined Dosage Generic Form by Reversed Phase High Performance Liquid Chromatography

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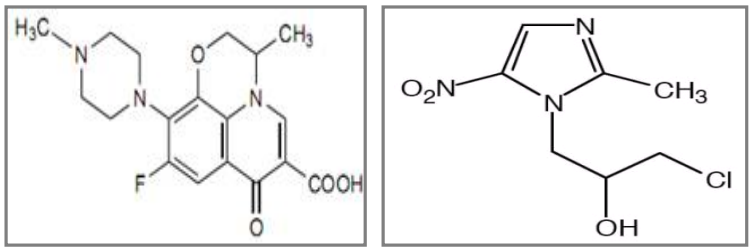
ABSTRACT

A new, sensitive, precise, and accurate validated reversed-phase-high performance liquid chromatographic assay method was reported for levofloxacin and ornidazole in combined dosage generic form. The present assay was developed using of C18 column (Inertsil 5µ, 250 mm × 4.6 mm) using the mobile phase potassium dihydrogen phosphate (pH 6.2) and acetonitrile (65:35%v/v)] at a flow rate was 1.0mL min^{-1} with UV detection wavelength of 300nm at ambient temperature. The developed RP-HPLC method was validated as per International Conference on Harmonization (ICH) guidelines with respect to specificity, limit of detection, limit of quantification, precision, linearity, accuracy, robustness and system suitability. The proposed reversed-phase-high performance liquid chromatographic method was found to be simple, sensitive and reproducible and can be used in routine analysis for simultaneous determination of levofloxacin and ornidazole in other brands of combined dosage forms.

Graphical Abstract



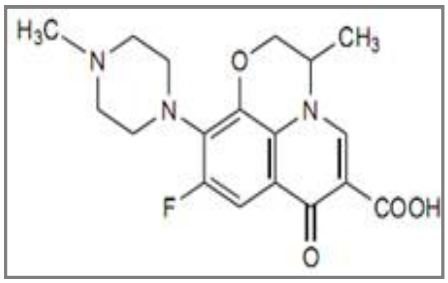
Chemical structure of Levofloxacin and Ornidazole

Keywords: Levofloxacin, Ornidazole Reversed-phase-high performance liquid chromatographic method and International Conference on Harmonization (ICH).

INTRODUCTION

Levofloxacin (-)-(S)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl]-7-oxo-7-H-Pyridol [1,2,3-di]-1,4-benzoxazine-6-carboxylic acid hemihydrate, is an third-generation fluoro quinolone antibiotic used to treat bacterial infections of the skin, sinuses, kidneys, bladder, or prostate [**1-6**].

Moreover, it is also used to treat bacterial infections causing bronchitis or pneumonia (Figure 1). Ornidazole [7-11], 1-chloro-3-(2-methyl-5-nitro-1H-imidazol-1-yl) propan-2-ol is an imidazole derivative used to treat protozoan infections (Figure 2).



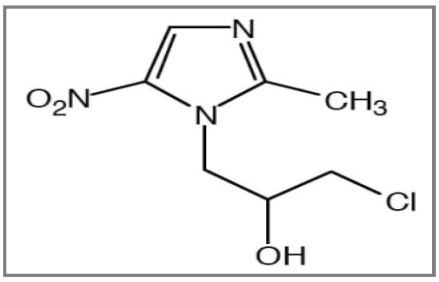


Figure 1. Chemical structure of Levofloxacin **Figure 2.** Chemical structure of Ornidazole

Combination of these two antibiotics (Levofloxacin and Ornidazole) is used in the treatment of diarrhea and dysentery of which levofloxacin toils by preventing the bacterial cells from dividing and repairing, thereby killing the bacteria and where as ornidazole kills parasites and anaerobic bacteria that cause infections by damaging their DNA of their immense therapeutic applications, various generic formulations of levofloxacin and ornidazole are in available at local pharmacy under several brands, i.e, FYNAL-OZ by Mankind Pharma Pvt Ltd, LEVOFLOX-OZ by Cipla Ltd etc label claim of Levofloxacin 250 mg and Ornidazole of 500 mg respectively.

Literature survey revealed seven HPLC methods [12-18] are reported for the determination of levofloxacin and ornidazole in combined dosage forms resulted in long retention times and low sensitivity. In this state, it made the author to develop and validate a new RP-HPLC method for the determination of the above said drugs in combined dosage generic forms as per ICH guidelines.

MATERIALS AND METHODS

Instrumentation: The present **c**hromatographic separation was carried on a PEAK chromatographic system equipped with LC-P7000 isocratic pump; Rheodyne injector with 20µL fixed volume loop, variable wavelength programmable UV detector UV7000 and the output signal was monitored and integrated by PEAK Chromatographic Software version 1.06. Tec comp UV-2301 double beam UV-Visible spectrophotometer was used to carry out spectral analysis and the data was recorded by Hitachi software. Sonicator (1.5L), Ultrasonicator was used to sonicating the mobile phase and samples. Standard and sample drugs were weighed by using Denver electronic analytical balance (SI-234) and pH of the mobile phase was adjusted by using Systronics digital pH meter.

Chemicals: Original samples of levofloxacin and ornidazole (99% pure) were obtained as gifted samples from Mankind Pharma Pvt Ltd. The purity of the above cited drug was evaluated by obtaining its melting point and ultraviolet (UV) and infrared (IR) spectra and were used without further purification.

Acetonitrile (HPLC grade), potassium dihydrogen phosphate and sodium hydroxide (AR grade) were purchased from Merck India and were used as obtained. All the dilutions were performed in standard class-A, volumetric glassware. For the assay of commercial formulation, FYNAL-OZ by Mankind Pharma Pvt Ltd (Containing 250 mg and 500 mg label claims of levofloxacin and ornidazole) were procured from the local market. Milli-Q water was used throughout the analysis.

Mobile phase preparation: Prepare a filtered and degassed mixture of potassium dihydrogen phosphate (pH 6.2) and acetonitrile (65:35%v/v) respectively.

Buffer preparation: Prepared by dissolving accurately potassium dihydrogen phosphate (pH 6.2) was prepared by dissolving 4.08 g of potassium dihydrogen phosphate in approximately 950 mL distilled water. The pH was adjusted to 6.2 with sodium hydroxide, and finally water was added to 1000 mL, Filtered through a 0.45 µ membrane filter.

Diluent Preparation: In the present assay mobile phase was used as diluent.

Standard solution: Weigh and transfer 10mg of levofloxacin and 100 mg of ornidazole standard into a 100mL of volumetric flask, containing sufficient amount of diluent, sonicated to dissolve and finally made up to volume with diluent to give a primary stock standard solution containing concentrations of 100µg mL^{-1},1000µg mL^{-1} for levofloxacin and ornidazole respectively. From the primary stock solution six working standard solutions of concentrations covering the range of 25-75 µg mL^{-1} for levofloxacin and 50-150 µg mL^{-1} for ornidazole were prepared by transferring and diluting different aliquots into a series of 10 mL volumetric flasks with the same diluent.

Sample Solution: Weighed and transferred 10 oral tablets of FYNAL-OZ by Mankind Pharma Pvt Ltd (Containing 250 and 500 mg label claims of levofloxacin and ornidazole) into a mortar and pestle. Crush the above tablets to fine powder. Weigh and transfer sample powder quantity equivalent to 10 mg of levofloxacin and 100 mg of ornidazole into a 100 mL volumetric flask containing 50mL of mobile phase and shaken vigorously, sonicated for 15 min and volume made up to the mark with mobile phase (Concentrations of 100 µg mL,1000 µg mL^{-1} for levofloxacin and ornidazole). Various aliquots of the above stock solution were pipetted and transferred into a series of cleaned, dry 10 mL volumetric flasks and the diluent was added up to the mark to get final concentration of 25-75 µg mL^{-1} for levofloxacin and 50-150 µg mL^{-1} for ornidazole respectively. 20 µL volume each of these standard and sample solutions were injected five times and the peak areas were recorded.

RESULTS AND DISCUSSION

Method development: In developing the present HPLC assay method, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all other conditions constant that include the selecting the appropriate wavelength and choice of stationary and mobile phases.

Selection of Detection wavelength: For this of solutions of levofloxacin and ornidazole containing 100% level concentration were prepared in mobile phase and the above prepared solution was scanned on UV spectrophotometer between 200-400 nm using diluent as blank and the λ_{max} was recorded separately. The λ_{max} was found to be 300 and 310 nm for levofloxacin and ornidazole respectively and the isosbestic point of levofloxacin and ornidazole was found to be 300nm as the both drugs gave maximum response at this wavelength**.**

Choice of stationary phase: Preliminary development trials were performed with C18 columns of different types, configurations and from different manufacturers. Finally, the excellent separation and peak shapes were obtained on reversed phase C18 column (Inertsil 5µ, 250 mm × 4.6 mm).

Selection of mobile phase: To attain sharp peaks, low tailing factor and good base line separation for the present components, a number of experiments were carried out by varying the composition of various solvents in the mobile phase. Mixtures of different organic solvents like methanol, Acetonitrile with mentioned buffer indifferent combinations were tested as mobile phases on the above described reversed phase C18 column (Inertsil 5µ, 250 mm × 4.6 mm). A mixture of potassium dihydrogen phosphate (pH 6.2) and acetonitrile (65:35 %v/v) was proved suitable with respect to all the combinations since, the chromatographic peaks obtained were better defined and resolved and were almost free from tailing.

Selection of Flow rate: In addition to the above parameters the flow rate of the mobile phase into the column was studied by changing the flow rates from 0.5–1.5 mL min^{-1} for optimum separation. It was found from the experiments that 1.0 mL min^{-1} flow rate was ideal for the successful elution of the above analytes. The trails and the results obtained during the method development carried by the author are deduced in table 1.

Table 1. Results of method development trails

Trial No	HPLC Conditions	Remarks
1	Flow : 1.0 mL min^{-1} Column: Inertsil 5µ, 250 mm × 4.6 mm Mobile Phase : Potassium dihydrogen phosphate (pH 6.2) and Acetonitrile (50:50%v/v)	The peaks of both levofloxacin and ornidazole were eluted and were not well resolved. Therefore, these chromatographic conditions were not suitable.
2	Flow : 1.0 mL min^{-1} Column : Inertsil 5µ, 250 mm × 4.6 mm Mobile phase : Potassium dihydrogen phosphate (pH 6.2) and Acetonitrile (60:40 %v/v)	The peaks were observed for both the drugs and they were not well resolved and hence these conditions were not suitable.
3	Flow : 1.0 mL min^{-1} Column: Inertsil 5µ, 250 mm × 4.6 mm Mobile phase : Potassium dihydrogen phosphate (pH 6.2) and Acetonitrile (65:35 %v/v)	Two peaks were observed in these conditions and peaks that are asymmetric free from tailing.

Chromatographic Conditions: Method development trails resulted in excellent separation of levofloxacin and ornidazole was on using C18 column (Inertsil 5µ, 250 mm × 4.6 mm) with mobile phase (potassium dihydrogen phosphate (pH 6.2) and acetonitrile (65:35 %v/v)) at a flow rate was 1.0 mL min^{-1} with UV detection wavelength of 300 nm at ambient temperature. The retention time for levofloxacin and ornidazole were found to be 3.213 and 5.446 min respectively (Figure 3 and Table 2).

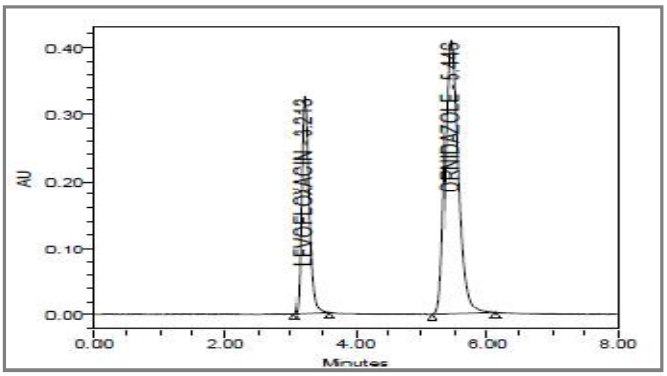


Figure 3. Typical Chromatogram of Standard.

Table 2. Optimized chromatographic conditions

S.No.	Parameter	Value
1	Column	C18 column (Inertsil 5µ, 250 mm × 4.6 mm)
2	Mobile phase	potassium dihydrogen phosphate (pH 6.2) and Acetonitrile (65:35%v/v)
3	Flow rate	1.0 mL min^{-1}
4	Diluent	Mobile phase
5	Column temperature	25°C
6	Runtime	8 min
7	Retention time	Levofloxacin--3.213min; Ornidazole- 5.446 min
8	Volume of injection	20 µL min^{-1}
9	Detection wavelength	300 nm

Method validation: The developed RP-HPLC method is further validated in accordance with ICH guidelines [**19**] for the assay of levofloxacin and ornidazole using the following parameters.

Specificity: The specificity of the proposed method was tested against standard compounds in the presence of blank and placebo under optimized test conditions. The comparison of the chromatograms of blank and placebo mixture revealed that there were no coeluting peaks with the peaks obtained for levofloxacin and ornidazole in the sample solution. Moreover, no interference from the blank and placebo was observed at the retention time of the levofloxacin and ornidazole respectively concluding that the proposed method is specific (Table 3).

Table 3. Specificity studies for levofloxacin and ornidazole

S. No.	Sample Name	Levofloxacin Area	Rt	Ornidazole Area	Rt
1	Standard	2625907	3.213	5649133	5.446
2	Sample	2644704	3.214	5583003	5.448
3	Blank	-	-	-	-
4	Placebo	-	-	-	-

System suitability: System suitability parameters like number of theoretical plates, HETP and peak tailing were determined for levofloxacin and ornidazole by using the above defined chromatographic conditions and the values for the parameters were within the acceptance criteria and are reported in table 4.

Table 4. System suitability studies of levofloxacin and ornidazole

Parameter	Levofloxacin	Ornidazole
Retention time	3.214	5.448
Theoretical plates	3728	3623
Tailing factor	1.40	1.30
% RSD	0.5	0.7

Linearity: The linearity of the proposed method was evaluated by analyzing working standard solutions of levofloxacin and ornidazole of five different concentrations. Twenty microliters of each solution of the above said concentrations were injected into the prescribed chromatographic system under operating chromatographic conditions described above. The chromatograms obtained for each concentration of the drug solution were recorded and the peak areas were determined.

Separate calibration curves of levofloxacin and ornidazole were obtained by plotting the peak area ratio determined versus the applied concentrations of levofloxacin and ornidazole (Figures 4 and 5).

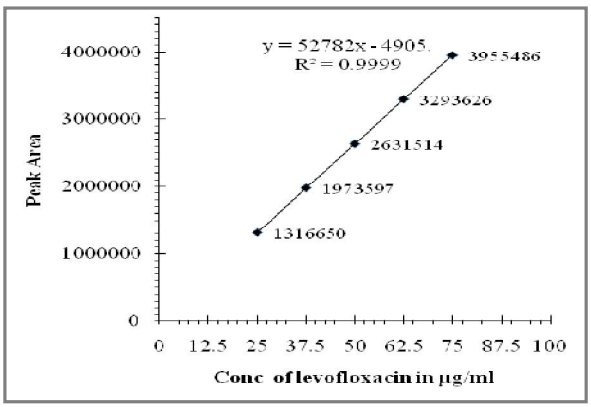


Figure 4. Linearity plot for levofloxacin.

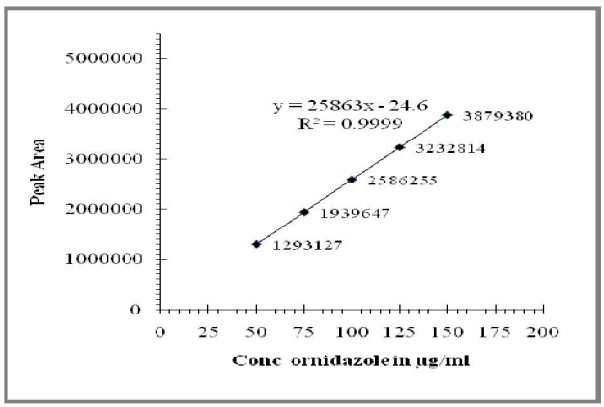


Figure 5. Linearity plot for ornidazole.

The linearity of the calibration graphs was validated by the high values of correlation coefficients, intercept and slope values of 4905.8-52781.61x (R^2=0.9999) for levofloxacin and Y = 24.6-25863x (R^2=0.9999) for ornidazole respectively (Table 5).

Detector response: The LOD of levofloxacin and ornidazole were found to be 0.0018 µg mL^{-1} and 0.0059 µg mL^{-1}, respectively and the LOQ values of levofloxacin and ornidazole were 0.0016 µg mL^{-1} and 1.97 µg mL^{-1} and are reported in table 6 respectively.

Table 5. Results of linearity studies of levofloxacin and ornidazole

Linearity of Levofloxacin		Linearity of Ornidazole	
Concentration (µg mL^{-1})	Peak Area	Concentration (µg mL^{-1})	Peak Area
25.0	1316650	50	1293127
37.5	1973597	75	1939647
50.0	2631514	100	2586255
62.5	3293626	125	3232814
75.0	3955486	150	3879380
Regression equation: Slope, b Intercept	Y = 4905.2-52781.5x (R^2=0.9999) :52781.6 a:-4905.8	Regression equation: Slope, Intercept	Y = 7327.2-55810.8x (R^2=0.9999) b:25863 a:-24.6

Table 6. LOD and LOQ Values (µg/ml) of levofloxacin and ornidazole

Parameter	Levofloxacin µg mL^{-1}	Ornidazole µg mL^{-1}
LOD	0.0018	0.0059
LOQ	0.016	1.97

Precision: The precision studies of the proposed method were ascertained by replicate analysis (Intra and inter-day precision studies) of tablet powder and the results were tabulated in table 7. The Intra - day precision studies for six sample preparations (100% Conc.) showed a %RSD of 0.10 for levofloxacin and 0.031 for ornidazole respectively revealing the high precision of the proposed RP-HPLC method.

Table 7. Results of precision studies of levofloxacin and ornidazole

Levofloxacin			Ornidazole		
S.No.	RT	Area	S.No.	RT	Area
Injection1	3.214	2634654	Injection1	5.443	5595208
Injection2	3.208	2634542	Injection2	5.437	5592964
Injection3	3.207	2636515	Injection3	5.434	5591030
Injection4	3.206	2639007	Injection4	5.434	5593038
Injection5	3.205	2632369	Injection5	5.439	5590331
Injection6	3.205	2631935	Injection6	5.436	5593658
*Mean		2634837	*Mean		5592705
*Std. Dev.		2639.611	*Std. Dev.		1777.228
*% RSD		0.10	*% RSD		0.031

***** Average of six determinations; SD=Standard Deviation; %RSD=Relative standard deviation

Accuracy: The accuracy of the present proposed method was performed at three levels, in which sample stock solutions were spiked with standard drug solution containing 50, 100 and 150% of labeled amount of both levofloxacin and ornidazole in generic tablets. Three replicate samples of each concentration level were prepared and the % recovery at each level (n=3), was determined and reported in table 8. The % of recovery was ranged from 98.72% to 99.15% for levofloxacin and 99.60% to 99.73% for ornidazole respectively revealing good accuracy.

Table 8. Results of accuracy studies of levofloxacin and ornidazole

Levofloxacin						Ornidazole					
S.No.	Accuracy level	Sample name	µg mL^{-1} added	µg mL^{-1} found	*% Recovery	S.No.	Accuracy level	Sample name	µg mL^{-1} added	µg mL^{-1} found	% Recovery
1	50%	1	24.750	24.71	98.72	1	50%	1	50.000	49.77	99.60
		2	24.750	24.69				2	50.000	49.74	
		3	24.750	24.64				3	50.000	49.90	
2	100%	1	49.500	49.59	99.15	2	100%	1	100.000	99.71	99.73
		2	49.500	49.58				2	100.000	99.80	
		3	49.500	49.56				3	100.000	99.68	
3	150%	1	74.250	74.26	99.03	3	150%	1	150.000	149.60	99.72
		2	74.250	74.33				2	150.000	149.59	
		3	74.250	74.31				3	150.000	149.60	

*Average of three determinations

Robustness studies: The robustness of the proposed method was verified by making deliberate changes to some parameters such as the mobile phase volume ratio, pH of the solution and detection wavelength. The factors selected in the present study were the change in the mobile phase volume ratio, change in flow rate by ±0.2ml/min and the change in temperature by ±5°C respectively. The developed method was found to be robust enough that the peak areas of levofloxacin and ornidazole were not apparently affected by small variation in the chromatographic conditions. The system suitability parameters were within the limits and shown in table 9 respectively.

Table 9. Results of Robustness studies of levofloxacin and ornidazole

Levofloxacin				Ornidazole			
Parameter	Rt	Theoretical Plates	Asymmetry	Parameter	Rt	Theoretical Plates	Asymmetry
Decreased flow rate (0.8 mL min^{-1})	3.217	3414	1.34	Decreased flow rate (0.8 mL min^{-1})	5.467	3390	1.29
Increased flow rate (1.2 mL min^{-1})	3.207	3260	1.36	Increased flow rate (1.2 mL min^{-1})	5.432	3310	1.29
Decreased temperature (20°C)	3.207	3497	1.35	Decreased temperature (20°C)	5.416	3432	1.27
Increased temperature (30°C)	3.200	3517	1.39	Increased temperature (30°C)	5.391	3503	1.31

Ruggedness: The ruggedness of the proposed RP-HPLC method was evaluated by a different analyst and different analyst and different instrument in the same laboratory. The % RSD for peak areas of levofloxacin and ornidazole was calculated and the experimental results are shown in table 10 and

Table 10. Results of Ruggedness studies of levofloxacin and ornidazole

Levofloxacin			Ornidazole		
S.No.	Rt	Area	S.No.	Rt	Area
Injection1	3.213	2625907	Injection1	5.446	5.446
Injection2	3.216	2650477	Injection2	5.442	5.442
Injection3	3.209	2647891	Injection3	5.437	5.437
Injection4	3.197	2649000	Injection4	5.422	5.422
Injection5	3.207	2622161	Injection5	5.431	5.431
Injection6	3.205	2634236	Injection6	5.443	5.446
*Mean		2638279	*Mean		5594935
*Std. Dev.		12532.55	*Std. Dev.		35003.56
*% RSD		0.475	*% RSD		0.625

*Average of six determinations, SD=Standard Deviation, %RSD=Relative standard deviation

these results revealed that the %RSD (0.475 and 0.625% of levofloxacin and ornidazole) was within the limits indicating that the developed RP-HPLC method was found to be rugged.

Analysis of marketed formulation: Analysis of marketed tablets [FYNAL-OZ oral tablets] was carried out using the above said optimized mobile phase and HPLC conditions. The % content of levofloxacin and ornidazole in FYNAL-OZ oral tablets (Containing 250 mg and 500 mg label claims of levofloxacin and ornidazole) were calculated and were found to be 98.35 and 98.72% respectively. Statistical t -test and f- test values of 0.398, 2.133 and 2.194, 1.15 for levofloxacin and ornidazole in dosage forms thereby concluding the proposed method extremely accurate (acceptance level of 95% to 100%) with respect to the reference method [**18**] reported. The results are given in table 11.

Table 11. Assay results of levofloxacin and ornidazole in formulations

Drug Name [Fynal-Oz Oral Tablets]	Quantity Label Claim(mg)	**Quantity Found(mg) ± SD	*% Assay ± %RSD	*% Assay ±RSD Reported Method[18]	t-Test	F-Test
Levofloxacin	250	246.62 ± 3.12	98.64 ± 0.41	98.35 ± 1.84	0.398	2.133
Ornidazole	500	493.98 ± 7.946	98.72± 1.50	100.76 ± 1.64	2.194	1.15

****** Average of six determinations; SD=Standard Deviation; the t-and F-test values refer to comparison of the proposed method with the reference method. Theoretical values at 95% confidence limit, F=5.05, t =2.262

APPLICATION

The proposed method eluted the present studied compounds in relatively short run time (< 5min) allowing to quantifying large number of samples in routine and quality control analysis by pharmaceutical labs. In continuation the proposed method can be employed a statistical experimental design as such to reduce cost of analysis and to increase sample throughput during routine analysis.

CONCLUSION

The method presented in this paper describes the development of a selective, accurate and sensitive RP-HPLC method for the simultaneous estimation of levofloxacin and ornidazole in pure and marketed formulations with good resolution. The proposed method when applied to the pharmaceutical dosage form obtained results that are in good agreement with the claimed amount of levofloxacin and ornidazole by the manufacturer. The validation results demonstrated that the proposed RP-HPLC procedure is suitable for the intended purpose and can be employed easily used for the routine quality control of levofloxacin and ornidazole in combined dosage forms within a short analysis time.

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