Analytical Method Development and Validation by RP-HPLC for the Simultaneous Estimation of Ornidazole and Ofloxacin in Pharmaceutical Dosage Form

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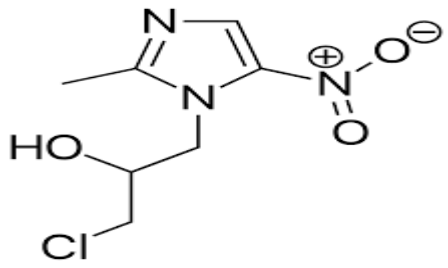
Abstract: ***A simple, precise, accurate and precise reverse phase high performance liquid chromatographic method has been developed and validated for the estimation of Ornidazole and Ofloxacin simultaneously in combined dosage forms. A Lichrospher 100 C-18 and mobile phase comprises of 750 volume of Water, 250 volume of Acetonitrile, 3.5 volume of Triethylamine, and the final pH adjusted to 3.25 ± 0.10 with 10% v/v o-phosphoric acid. Measurements were made at the effluent flow rate of 1.0 ml/min with injection volume 20 µl and ultraviolet (UV) detection at 320 nm, as both components show reasonably good response at this wavelength. The retention times of Ornidazole and Ofloxacin were 5.75 min and 3.25 min, respectively. The method was validated in terms of linearity, accuracy, precision, robustness and specificity. Linearity of Ornidazole and Ofloxacin was in the range of 1-70 µg/ml and 1-70 µg/ml, respectively. The limit of detection and limit of quantification were found to be 0.3 and 0.9 mg/ml for Ornidazole and, respectively and for Ofloxacin were 0.5 and 1.52 mg/ml respectively. The method was validated for specificity, linearity, precision, accuracy and robustness. The linear regression analysis data, Limit of Detection values, Limit of Quantitation values and Percentage RSD of Ornidazole and Ofloxacin were found under acceptance criteria. The method is useful in the quality control of bulk manufacturing and pharmaceutical dosage forms.***

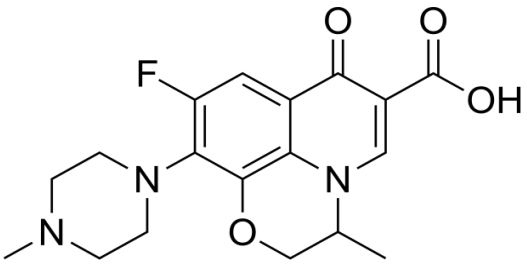
Key Words: Ofloxacin; Ornidazole, RP-HPLC; Validation.

INTRODUCTION

Ofloxacin is a member of the fluoroquinolone class of antibacterial. Chemical name of ofloxacin is 9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylic acid. Ofloxacin is bactericidal and acts by inhibiting the A subunit of DNA gyrase (topoisomerase) which is essential in the reproduction of bacterial DNA. Ofloxacin is more active than ciprofloxacin against *Chlamydia trachomatis*. It is also active against *Mycobacterium leprae* as well as *M. Tuberculosis* and some other Mycobacterium species. Chemical name of ornidazole is α- (Chloromethyl)-2-methyl-5-nitroimidazole-1-ethanol. Ornidazole is a nitroimidazole agent indicated in the treatment of infections such as trichomoniasis, amebiasis, and giardiasis. In the present investigation an attempt has been made to develop accurate and precise HPLC method for the simultaneous estimation of Ornidazole and Ofloxacin in combined dosage forms.



Structure of Ornidazole



Structure of Ofloxacin

EXPERIMENTAL

Instrumentation

- ❖ Merck - Hitachi isocratic High Performance Liquid Chromatography system comprising of,
  - ➢ Hitachi pump L - 7110
  - ➢ Rheodyne universal injector 77251 with injection volume 20 µl
  - ➢ Hitachi L - 7420 UV - Visible Detector
  - ➢ Merck - Hitachi HSM software

- LiChrospher® 100 rp-180, C_{18}, column having 250 mm length, 4.0 mm internal diameter and 5 µm particle size.

❖ Shimadzu model 1601 double beam UV - Visible Spectrophotometer with a pair of 10 mm matched quartz cells.
❖ Shimadzu – libror 220 balance
❖ Ultrasonic bath (Frontline Fs 4 ultrasonic cleaner)
❖ Digital pH meter (Analab)
❖ Corning volumetric flasks (10, 25, 50, 100, 250 ml)

Chemicals and materials

- Ornidazole (OZ) and Ofloxacin (OFX) bulk powders were kindly gifted by Excel Laboratories, Mehsana, India.
- Acetonitrile, Water used was of HPLC grade. (Rankem)
- Triethylamine and *o*-phosphoric acid (AR grade, S.D. Fine Chemicals Ltd., Mumbai)
- Combined suspension of Ornidazole and Ofloxacin were procured from local market.

Chromatographic conditions

The chromatographic separations were performed using LiChrospher® 100 C_{18}, 5 µm, 250 × 4.0 mm i.d. column, at ambient temperature.
The elution was monitored at 320 nm.
The injection volume was 20 µl.
Flow rate of 1.0 ml/min.
The mobile phase was filtered through nylon 0.45 µm- 47 mm membrane filter and was degassed before use.

Preparation of the mobile phase

The mobile phase was prepared by mixing 750 volume of Water, 250 volume of Acetonitrile, 3.5 volume of Triethylamine, and the final pH adjusted to 3.25 ± 0.10 with 10% v/v *o*-phosphoric acid.
Flow rate of 1.0 ml/min.

Preparation of standard stock solutions

Standard OZ stock solution (1 mg/ml)

Accurately weighed OZ (25.0 mg) was transferred to a 25 ml volumetric flask. Dissolve and dilute to volume with diluents and mix.

Standard OFX stock solution (1 mg/ml)

Accurately weighed OFX (25.0 mg) was transferred to a 25 ml volumetric flask. Dissolve and dilute to volume with diluents and mix.

Mixed standard stock solution of OZ and OFX (100 µg/ml)

10 ml aliquots from each stock solutions of OZ and OFX were transferred and mixed in 100 ml volumetric flask and volume was made up with mobile phase up to mark to get 100 µg/ml mixed standard stock solution.

Bulk powder

Accurately weighed OZ (25 mg) and OFX (10 mg) was transferred to a 100 ml volumetric flask and dissolved in and diluted to mark with water. The solution (2.0 ml) was transferred to a 10 ml volumetric flask and diluted to the mark with mobile phase to obtain final solution with OZ (50 µg/ml) and OFX (20 µg/ml).

Sample solution

20 tablets were weighed, their average weight was determined, and crushed in mortar. An amount of powdered mass equivalent to 10 mg of OFX and 25 mg of OZ was weighed and transferred in 100 ml volumetric flask and mixed with 50 ml of water. To ensure complete extraction of drugs it was sonicated for 30 min. The solution was filtered through Whatman filter paper No. 41 and the residue was washed thoroughly with 10 ml water. The filtrate and washings were combined in a 100 ml volumetric flask and diluted to the mark with water. 2 ml aliquot from above solution was transferred in 10 ml volumetric flask and volume was adjusted with mobile phase up to mark to achieve sample solution with OFX (20 µg/ml) and OZ (50 µg/ml).

Determination of wavelength of maximum absorbance

The standard solutions of OZ and OFX were scanned in the range of 200-400 nm against mobile phase as a blank. OZ and OFX showed reasonably good absorbance at 320 nm.

Solution stability

Sample solutions were kept at 25^0C and 2-8^0C for 24 h and 3 days, respectively. Assay of initial time period was compared with these two time points. The falls in the assay values were evaluated. The difference between assays should not be more than 2 % for formulation, and 0.5% for Active Pharmaceutical Ingredients.

The plots of peak area verse the respective concentration of OZ and OFX were found to be linear in the range of 1-70 µg/ml for both the drugs,

with co-efficient of correlation, (r^2) 0.9963 and 0.9985, respectively.

Selection of Mobile phase and column

Reverse Phase Liquid Chromatography (RPLC) is suitable for the simultaneous analysis of OZ and OFX[1,3]. In RPLC, liChrospher® 100 rp-180, C_{18}, column having 250 mm length, 4.0 mm internal diameter and 5 µm particle size was used.

Resolution is the most important criteria for the method, and is imperative to achieve good resolution among the both compounds. As per the value of Ka and solubility of both the compounds, various compositions of mobile phase with different pH ranges (2.75 to 7.0) were tried and best resolution was obtained with mobile phase consisting of water, acetonitrile and triethylamine in the proportion of 750 volume of Water, 250 volume of Acetonitrile, 3.5 volume of Triethylamine with finally pH adjusted 3.25 ± 0.10 with 10 % v/v *o*-phosphoric acid.

A representative chromatogram is shown in Figure 1and 2 Parameters of chromatogram are shown in Table 1. Better resolution of the peaks with clear base line separation was found.

Retention time for OZ and OFX was 5.75 min and 3.25 min, respectively. Asymmetric factor for OZ and OFX was 1.35 and 1.636, respectively. The values of tailing factor for OZ and OFX was 1.12 and 1.678, respectively. Hence both the drugs are better resolved and separated with above mentioned mobile phase. Quantification was achieved with UV detection at 320 nm based on peak area.

VALIDATION OF THE PROPOSED METHOD

SPECIFICITY

Specificity The peak purity of OZ and OFX were assessed by comparing the retention time (TR) of standard OZ and OFX. Good correlation was also found between the retention time of standards and sample of OZ and OFX. (Figure 3 and Table 1)

LINEARITY AND RANGE

Appropriate aliquots from standard stock solution of mixed drugs were suitably diluted with mobile phase in such a way to get concentrations in a range of 1-70 µg/ml for both drugs. These solutions (n=5) were injected in to the universal injector 77251 (Rheodyne) with injection volume 20 µl. Evaluation of two drugs was performed with UV/Visible detector at 320 nm. Peak areas were recorded for all the peaks. The linearity of the calibration graphs was validated by the high value of correlation coefficients of the regression equation (r^2 = 0.9963 for OZ and 0.9985 for OFX). Characteristic parameters for regression equation and correlation are given in Table 2.

LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

The limit of detection and the limit of quantification of the drug were calculated using the following equations as per International Conference on Harmonization (ICH) guidelines.

$LOD = 3.3 \times \sigma/S$ and $LOQ = 10 \times \sigma/S$

Where, σ = standard deviation of the response

S = slope of the calibration curve

The limit of detection and the limit of quantification of the drugs were calculated. LOD for OZ and OFX were found to be 0.3 µg/ml and 0.5 µg/ml, respectively. LOQ for OZ and OFX were found to be 0.9 and 1.52 µg/ml respectively. (Table 2).

ACCURACY

It was carried out by recovery study using standard addition method. Known amount of standard OFX and OZ were added in to pre-analyzed sample and subjected them to the proposed HPLC method.

This was carried out to check the recovery of the drugs at different levels in the formulations i.e. multilevel recovery study. The pre analyzed samples were spiked with extra 25%, 50%, 100%, 150% and 200% of the standard OZ and OFX respectively, and the mixtures were analyzed by proposed method. The percent recoveries obtained for Ornidazole and for Ofloxacin was given in table 3.

PRECISION

Method precision (Repeatability)

Relative standard deviation of all the parameters is less than 2% in Table 4), which indicates that the proposed method is repeatable.

Intra-day and Inter-day precision:

It expresses within laboratory variations as on different days analysis or equipment within the laboratory. The intra- and inter-day variation for the determination of OZ and OFX were carried out at four different concentration levels 10, 20, 30 and 40 µg/ml.

The low % CV values of intra-day OZ and inter-day OFX precision revealed that the proposed method is precise.(Table 5a and 5b)

ROBUSTNESS

By introducing small changes, the flow rate by ± 0.2 ml and by changing the wavelength by ± 2nm, the effects on the results were examined. Robustness of the method was done at concentration levels 20 µg/ml with five times. System suitability was evaluated in each condition and sample was analyzed. The results were tabulated in table 6.

ASSAY OF THE MARKET FORMULATION

The proposed validated method was successfully applied to determine OZ and OFX in bulk powder and in tablet dosage forms. Results are given in Table 7. No interference of the excipients with the peaks of interest appeared; hence the proposed method is applicable for the routine simultaneous estimation of OZ and OFX in pharmaceutical dosage forms.

The results of the analysis of pharmaceutical dosage forms by the proposed method are highly reproducible, reliable and are in good agreement with the labeled claim of the drug. The % recoveries reveal that excipients usually present in the pharmaceutical formulations do not interfere.

RESULT

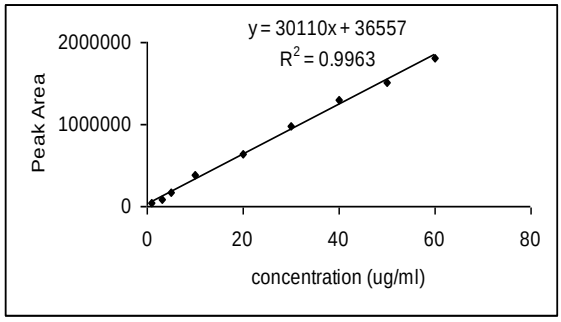


Figure 1. Calibration curve of Ornidazole

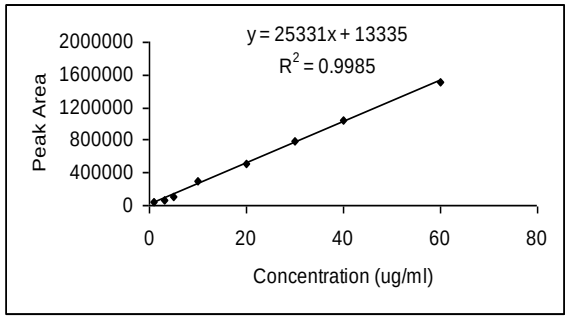


Figure 2. Calibration curve of Ofloxacin

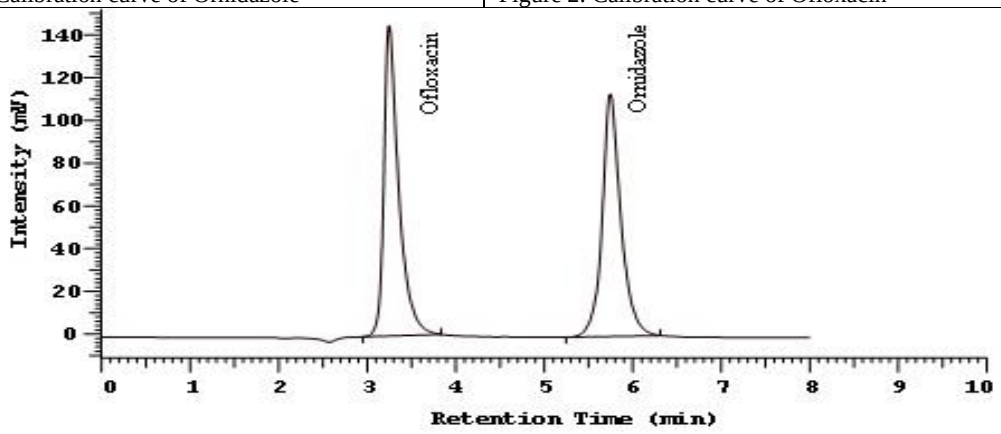


Figure 3. Chromatogram of OZ and OFX with corresponding retention time at 320 nm.

Table 1: System suitability parameters of chromatogram for OZ and OFX

Parameters	OZ	OFX
Retention time (min)	5.75	3.25
Tailing factor	1.12	1.678
Asymmetry	1.35	1.636
Theoretical plates	4358.5	2600.7

Table 2: Optical and regression characteristics for analysis of OFX and OZ by RP-HPLC method

Parameters	OZ	OFX
Concentration range (µg/ml)	1-70	1-70
Limit of Detection (LOD) (µg/ml)	0.3	0.5
Limit of Quantification (LOQ) (µg/ml)	0.91	1.52
Regression equation (y*= a + bc)		
Slope (b),	30110	25331
Intercept (a)	+36557	+13335
Regression coefficient (r^2)	0.9963	0.9985
y*= a + bc, where c is the concentration		

Table 3: Recovery data for the proposed method

Drug	Spike Level	Actual %	Assay %	%Recovery
Ornidazole	25%	25	24.95	99.80
	50%	50	49.24	98.48
	100%	100	99.35	99.35
	150%	150	149.15	99.44
	200%	200	195.27	97.64
Ofloxacin	25%	25	24.80	99.20
	50%	50	48.97	97.94
	100%	100	99.12	99.12
	150%	150	148.89	99.26
Ornidazole	Overall Mean			98.942
	Overall SD			0.87454
	Overall RSD			1.000398
Ofloxacin	Overall Mean			98.88
	Overall SD			0.6292
	Overall RSD			0.6364

Table 4: Method precision data for analysis of OZ and OFX by RP-HPLC method

OZ and OFX (20 µg/ml)	Retention time (min)		Peak area		Asymmetry		Tailing factor	
	OZ	OFX	OZ	OFX	OZ	OFX	OZ	OFX
1	5.75	3.25	638759	513015	1.35	1.64	1.12	1.67
2	5.77	3.31	649589	520789	1.33	1.61	1.13	1.64
3	5.72	3.24	640125	520000	1.36	1.65	1.13	1.64
4	5.81	3.23	641994	498641	1.32	1.66	1.14	1.71
5	5.73	3.25	651803	504781	1.36	1.63	1.13	1.68
6	5.72	3.24	647890	522648	1.39	1.64	1.09	1.7
Mean	5.75	3.25	645026.7	513312.3	1.35	1.64	1.12	1.67
SD	0.035	0.028	5430.273	9756.026	0.025	0.017	0.017	0.029
% CV	0.61	0.88	0.842	1.901	1.84	1.05	1.56	1.74

Table 5 a: Intra-day precision data for analysis of OZ and OFX by RP- HPLC method

Concentration		Intra-day precision			
OZ	OFX	OZ		OFX	
(µg/ml)	(µg/ml)	Mean ± S.D. (n=5)	% CV	Mean ± S.D (n=5)	% CV
10	10	380039 ± 6654.36	1.751	296787±3400.45	1.145
20	20	641994 ± 9783.45	1.524	513015 ± 8887.99	1.733
30	30	985382 ± 15740.31	0.533	782426 ± 5433.94	0.694
40	40	1302146± 22955.48	1.597	1051367 ±17391.47	1.655

Table 5 b: Inter-day precision data for analysis of OZ and OFX by RP- HPLC method

Concentration		Inter-day precision			
OZ	OFX	OZ		OFX	
(µg/ml)	(µg/ml)	Mean ± SD (n=5)	% CV	Mean ± SD (n=5)	% CV
10	10	380517 ± 1076.79	0.283	296679 ± 3643.87	1.229
20	20	641956 ± 4465.48	0.696	513188.4 ± 6460.55	1.259
30	30	985970.8 ± 3538.14	0.359	782112 ± 3773.60	0.483
40	40	1302088± 22387.64	1.721	1051126 ± 15908.83	1.514

Table 6. Data for Robustness

PARAMETER	OZ (20 µg/ml)		OFX (20 µg/ml)	
	MEAN ± S.D. (n=5)	% CV	MEAN ± S.D. (n=5)	% CV
Plus Flow (1.2 ml/min)	641899 ± 8978.45	1.398	513234 ± 7894.87	1.538
Minus Flow (0.8 ml/min)	6416345 ± 9894.34	0.155	512648 ± 7543.37	1.471
Plus Wavelength (322 nm)	642367 ± 8978.86	1.398	513678± 9647.68	1.878
Minus Wavelength (318 nm)	6414356 ± 8768.15	0.137	512675 ± 7594.19	1.482

Table 7: Application of proposed RP-HPLC method to the determination of tablets

Formulation	Drug	Labeled/taken amount (mg)	Amount found (mg)	% Amount found ± S.D. (n=5)
Bulk powder	Ornidazole	25	25.41	100.64± 1.23
	Oflaxacin	10	10.12	101.23 ± 0.85
Tablets	OFLA-OZ Ornidazole Ofloxacin	 500 200	 499.66 200.64	 99.94 ± 1.54 100.35 ± 0.73
	OFLOSTAR-OZ Ornidazole Ofloxacin	 500 200	 505.6 197.1	 101.02 ± 1.44 98.55 ± 0.72

CONCLUSION

The HPLC method which is developed for quantitative Simultaneous estimation of Ornidazole and Ofloxacin in combination in bulk and pharmaceutical dosage form. It is simple, economic, sensitive, precise, efficient and reproducible and is suitable for its intended purpose. The method was validated as per ICH guidelines, showing satisfactory analytical data for all the method validation parameters tested. Hence, the proposed method can be utilized for assessing the quantitative Simultaneous determination of Ornidazole and Ofloxacin in combination in bulk and pharmaceutical dosage form.

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