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Research Article

DEVELOPMENT AND VALIDATION OF A RP- HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF METRONIDAZOLE AND NORFLOXACIN IN BULK AND TABLET DOSAGE FORM

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ABSTRACT

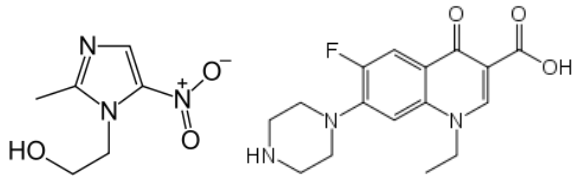
A simple reversed-phase high-performance liquid chromatographic (RP-HPLC) method has been developed and validated for simultaneous determination of metronidazole and norfloxacin in bulk and tablet dosage form. Chromatographic analysis was performed on a Hi Q Sil C_{18} column (250x 4.6 mm, 5µm) with Acetonitrile: 0.5 M potassium dihydrogen orthophosphate buffer pH 4.5 with triethylamine 30:70 (v/v) as mobile phase, at a flow rate of 0.9 ml/ min. UV detection was performed at 289 nm. The method was validated for accuracy, precision, specificity, linearity and sensitivity. The retention times of metronidazole and norfloxacin were 7.5 and 9.9 min, respectively. Calibration plots were linear over the concentration ranges 10-40 µg mL-1 and 8-32 µg mL-1 for metronidazole and Norfloxacin respectively. The Limit of detection was0.60 and 0.39g mL-1 and the quantification limit was 1.9g mL-1 and 1.08g mL-1 for metronidazole and norfloxacin respectively. The accuracy of the proposed method was determined by recovery studies and found to be 99.05% to 100.03%. Commercial tablet formulation was successfully analyzed using the developed method and the proposed method is applicable to routine analysis of determination of metronidazole and norfloxacin in bulk and tablet dosage form.

Keywords*:* Metronidazole, Norfloxacin, RP-HPLC, ICH guidelines.

INTRODUCTION

Metronidazole is used as an anti-protozoal, chemically it is 2-(2-methyl-5-nitro-1*H*- imidazol-1-yl) ethanol. Norfloxacin is used as an anti- bacterial agent; chemically it is 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-*1H*-quinoline-3- Carboxylic acid. A literature survey reveals that various analytical methods like metronidazole in dermatological formulations by

HPLC[1], Simple HPLC- UV method for determination of norfloxacin and metronidazole[2, 3, 4, 5] and its application to a comparative pharmacokinetic study in human volunteers[6], and a single method for the simultaneous estimation of metronidazole and norfloxacin by RP- HPLC[7] have been reported. There is need for an interest to develop simple, accurate, specific, sensitive, precise and reproducible simultaneous RP-HPLC method for the estimation of metronidazole and norfloxacin in bulk and its formulation.



(a) **(b)**

Fig. 1: It shows chemical structure of Metronidazole (a) and Norfloxacin (b)

MATERIALS AND METHODS

Metronidazole (METRO) and Norfloxacin (NOR) were gifted by Aarati Drugs Ltd., (Mumbai, India). All chemicals and reagents used were of HPLC grade for HPLC analysis and analytical grade for spectroscopic study are purchased from Loba Chemicals and Thomas chemicals (Mumbai, India). Commercial pharmaceutical preparation Nor-metrogyl which was claimed to contain 500mg of metronidazole and 400 mg of norfloxacin is used in analysis.

Instrumentation and Chromatographic Conditions

High performance liquid chromatography Jasco pump PU-2080 plus equipped with Jasco UV detector UV-2075 plus was used. Mobile phase comprising of Acetonitrile: 0.5 M potassium dihydrogen orthophosphate buffer pH 4.5 with triethylamine 30:70 (v/v) at flow rate of 0.9 ml min-1 was performed on C18 column (250x 4.6 mm, 5µm). The effluent was detected at 289 nm. The retention times of metronidazole and norfloxacin were 7.5 and 9.9 min. The Column temperature was maintained at ambient and the volume of injection was 10µL.

Preparation of mobile phase

The HPLC grade solvents were used for the preparation of mobile phase. Phosphate buffer (0.5 M) was prepared by dissolving accurately weighed quantity of 3400 mg of Potassium dihydrogen orthophosphate in a 500.0 ml of double distilled water. Mobile phase was prepared by mixing 30.0 ml of acetonitrile with 70.0 ml of 0.5 M Phosphate buffer (pH adjusted to 4.5 with triethylamine). This mobile phase was filtered through 0.42 µ membrane filter and then it was ultrasonicated for 30 minutes.

Standard solution

About 10 mg of each reference standard of METRO and NOR were accurately weighed & transferred to 100 ml volumetric flasks. Both the drugs were dissolved in 50 ml of mobile phase with shaking and then volume was made up to the mark with mobile phase to get 100 µg/ml of standard stock solution of each drug. Then it was ultrasonicated for 10 minutes and filtered through 0.20 µ membrane filter. Linearity was determined in the range of 10-40 µg mL-1 and 8-32 µg mL-1 for metronidazole and norfloxacin.

Assay in formulation

Twenty tablets containing 500 mg of METRO and 400 mg of NOR were weighed and average weight was calculated. The tablets were crushed and powdered in glass mortar. For the analysis of drugs, quantity of powder equivalent to 50 mg of METRO and 40 mg of NOR was transferred to a 50 ml volumetric flask containing 30 ml of mobile phase. (50mg: 40mg METRO: NOR) and then ultrasonicated for 20 min. Finally the volume was made up to the mark with mobile phase. The solution was filtered through Whatman filter paper No. 42. This solution was further diluted with mobile phase to obtain mixed sample solution containing 20 µg/ml of METRO and 16 µg/ml of NOR. The solution was filtered through 0.20 µ membrane filter. A 20 µl of sample solution was injected to into sample injector for six times under chromatographic condition as described above. Areas of each peak were measured at 289 nm. The amount of each drug

present in the sample was determined from peak area of METRO and NOR present in the pure mixture respectively.

RESULTS AND DISCUSSIONS

The proposed HPLC method required fewer reagents and materials and it is simple and less time consuming. This method could be used in quality control test in pharmaceutical industries. The chromatogram of metronidazole and norfloxacin were shown in (Fig.1). There was clear resolution between rosiglitazone and metformin with retention time of 7.5 and 9.9 minutes, respectively.

Linearity

The response was determined to be linear over the range of 10-40 µg mL^{-1}(10,15,20,25,30,35,40)for metronidazole and 8-32 µg mL^{-1} (8,12,16,20,24,28,32) for norfloxacin. The solutions were injected into HPLC system. Each of the concentration was injected in triplicate to get reproducible response. The run time was 14 min and the peak areas were measured. The calibration curve was plotted as concentration of the respective drug versus the response at each level. The proposed method was evaluated by its correlation coefficient and intercept value calculated by statistical study. They were represented by the linear regression equation (Fig 3 and 4 calibration curve).

Accuracy

To check the accuracy of the proposed method, recovery studies were carried out according to ICH guidelines by applying the standard addition method to known amount of METRO and NOR corresponding to 80, 100 and 120%. In 80% recovery study, amount of standard added to 1.5 ml tablet stock solution is 1.2 ml and 0.96 ml of METRO and NOR respectively. In 100% recovery study the amount of standard added is 1.5 ml and 1.2 ml of METRO and NOR respectively. In 120% recovery study the amount of standard added is 1.8 ml and 1.44 ml of METRO and NOR respectively. Finally the volume was made up to the mark with mobile phase. The recovery studies were performed three times at each level. The results of the recovery studies and its statistical evaluation are summarized in Table no.2 and Table no.3.

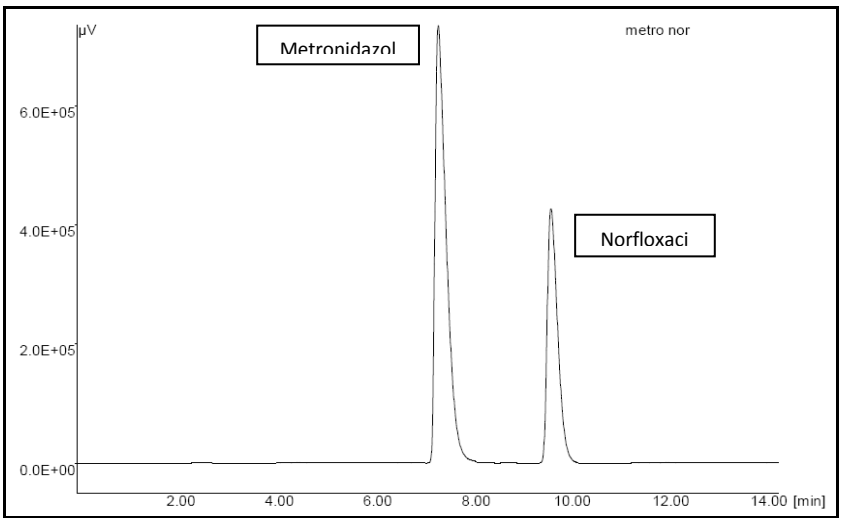


Fig. 2: It shows a typical chromatogram of Metronidazole and Norfloxacin

Table 1: Table shows peak area of Metronidazole and Norfloxacin

METRO		NOR	
Conc. (µg/ml)	**Mean peak area**	**Conc. (µg/ml)**	**Mean peak area**
10	411026	8	253095
15	823052	12	517190
20	1159078	16	739285
25	1644104	20	1010240
30	2056130	24	1275475
35	2386156	28	1494304
40	2877182	32	1771665

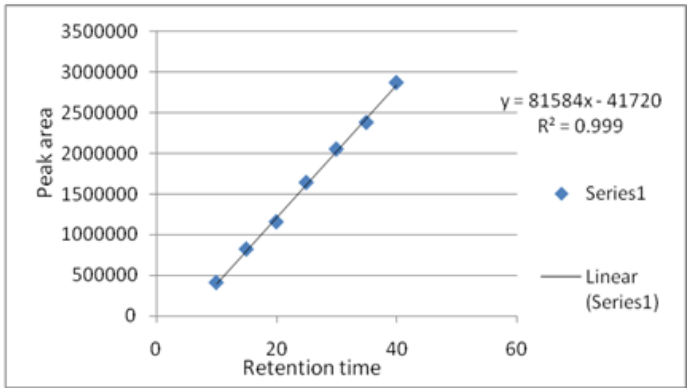


Fig. 3: It shows calibration curve of Metronidazole by HPLC

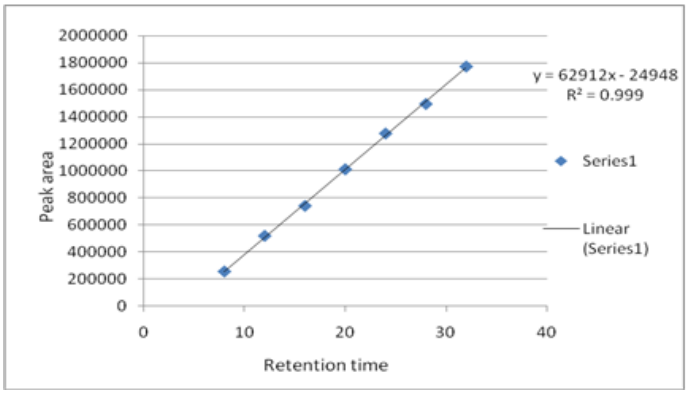


Fig. 4: It shows Calibration curve of Norfloxacin by HPLC

Table 2: Table shows Results of Recovery studies

Recovery Level	Drug	Conc.Of drug(µg/ml)		Total Conc of Drug	Total amt recovered (µg/ml)	% Recovery*
		Drug taken	Std drug added			
80		15	12	27	26.86	99.50
100	METRO	15	15	30	29.71	99.05
120		15	18	33	32.78	99.35
80		12	9.6	21.6	21.06	100.03
100	NOR	12	12	24	23.93	99.74
120		12	14.4	26.4	26.24	99.42

Limit of Detection and Quantification

Limit of detection is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. The detection limit (LOD) and quantitation limit (LOQ) may be expressed as:

L.O.D. = 3.3(SD/S)

L.O.Q. = 10(SD/S)

Where,

SD = Standard deviation of the response,

S = Slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The LOD was found to be 0.60g mL^{-1} and 0.39g mL^{-1} and LOQ was found to be 1.9g mL^{-1} and 1.08g mL^{-1} for metronidazole and norfloxacin respectively which represents that sensitivity of the method is high. Values of LOD and LOQ are shown in Table 3.

Table 3: Table shows LOD and LOQ of Metronidazole and Norfloxacin

Parameter	Metronidazole	Norfloxacin
LOD (µg/ ml)	0.60	0.39
LOQ (µg/ ml)	1.9	1.08

Precision

Repeatability involves analysis of replicates by the analyst using the same equipment and method and conducting the precision study over short period of time while reproducibility involves precision study at different occasions, different laboratories, and different batch of reagent, different analysts, and different equipments. To check the degree of repeatability of the method, six samples of the marketed formulations were analyzed.

Ruggedness

The ruggedness of an analytical method is determined by analysis of aliquots from homogenous lots by different analysts using operational and environmental conditions that may differ but are still within the specified parameters of the assay. The assay was performed in different condition, different analyst, and different dates. The results are given in Table 5.

Robustness

The robustness of the method was determined by deliberate changes in the method like alteration in pH of the mobile phase, percentage organic content, changes in the wavelength. The robustness of the method shows that there were no marked changes in the chromatographic parameters, which demonstrates that the method developed is robust.

Table 4: Table shows Statistical evaluation of marketed formulation

Drug	Amount of drug found	%Mean *	S.D.	%RSD	S.E.
METRO	19.923	99.61	0.4404	0.4417	0.2855
NOR	15.86	99.17	0.5588	0.5634	0.3794

Table 5: Table shows Results of ruggedness studies

Parameter	Drug	% Mean amt. estimated*	± S.D.*	%RSD*	S.E.
Analyst-I	METRO	99.61	0.4404	0.4417	0.2855
	NOR	99.17	0.5588	0.5634	0.3794
Analyst-II	METRO	100.035	0.5436	0.5434	0.3641
	NOR	99.05	0.4448	0.4491	0.2318

Table 6: Table shows Robustness evaluation of Metronidazole and Norfloxacin

Factor	Level	Retention time of METRO	Retention time of NOR
A: Flow Rate (ml/min)			
0.8	-1	7.8	10.5
0.9	0	7.5	9.9
1.0	+1	7.3	9.3
Mean ± S.D (n=6)		7.5 ±0.25	9.91 ±0.6
B: Percentage of Acetonitrile in the mobile phase (v/v)			
29	-1	7.6	9.7
30	0	7.5	9.9
31	+1	7.9	10.2
Mean ± S.D (n=6)		7.6 ± 0.20	9.94 ± 0.25
C: pH of mobile phase			
4.4	-1	7.3	9.6
4.5	0	7.5	9.9
4.6	+1	8	10.3
Mean ±S.D (n=6)		7.6± 0.36	9.93 ± 0.35

Specificity

The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the sample matrix. If an analytical procedure is able to separate and resolve the various components of a mixture and detect the analyte qualitatively the method is called selective. It has been observed that there are no peaks of diluents and placebo at main peaks. Hence, the chromatographic system used for the estimation of metronidazole and norfloxacin is very selective and specific .Specificity studies indicating that the excipients did not interfere with the analysis.

For demonstrating the specificity of the method for drug formulation the drug was spiked and the representative chromatogram is shown in figure 5.

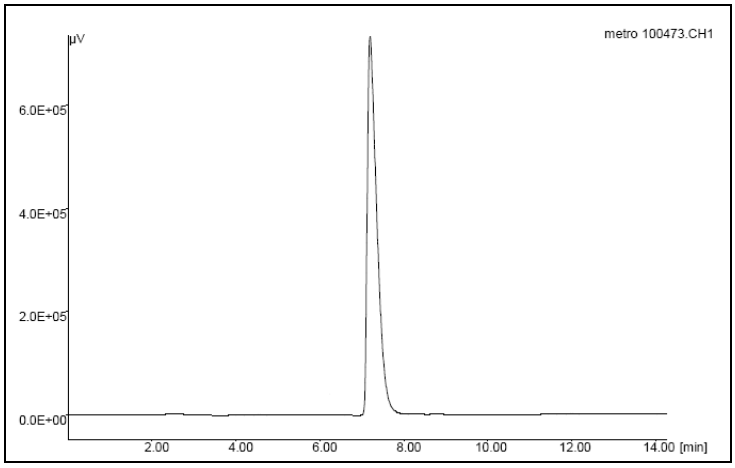


Fig. 6: It shows Chromatogram of Metronidazole showing specificity

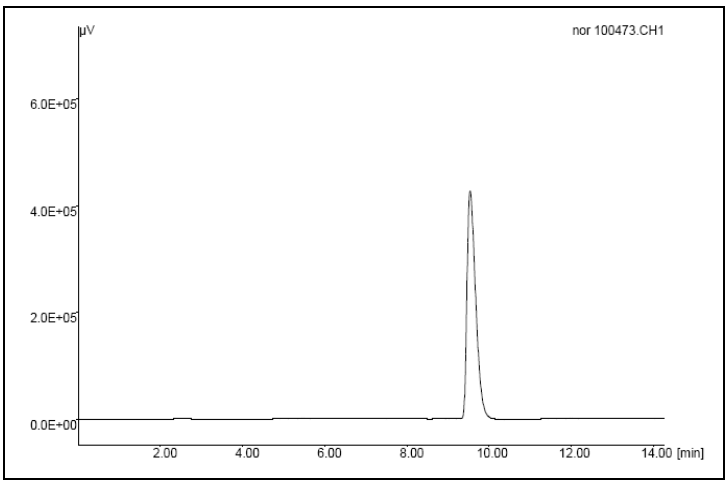


Fig. 7: It shows Chromatogram of Norfloxacin showing specificity

System Suitability

The values for system suitability parameters showed feasibility of this method for routine pharmaceutical application. Results are shown in the Table 7.

Table 7: Table shows System Suitability Parameters

S. No.	Parameters	METRO	NOR
1.	Retention time	7.5	9.9
2.	Resolution (R)	2.4	
3.	No. of theoretical plates (N)	2489	3834
4.	Tailing factor	0.60	0.54
5.	Selectivity (α)	1.3	

CONCLUSION

The proposed RP-HPLC method is found to be simple, accurate, precise, linear, and specific for quantitative estimation of metronidazole and norfloxacin in bulk and its formulation. The proposed RP-HPLC method is cost effective and less time consuming. The values for system suitability parameters showed feasibility of this method for routine pharmaceutical application. Hence the present HPLC method is suitable for routine assay of metronidazole and norfloxacin in raw materials and in pharmaceutical formulations in the quality control laboratories.

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