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Original Article

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS QUANTITATIVE ESTIMATION OF METRONIDAZOLE AND NALIDIXIC ACID IN TABLETS

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ABSTRACT

Objective: To develop an accurate, precise and linear Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method for simultaneous quantitative estimation of Metronidazole and Nalidixic acid in tablets and validate as per ICH guidelines.

Methods: The optimized method uses a reverse phase C18 column Inertsil ODS C18 column (250X4.6 mm×5µ) mobile phase consisting of mixed phosphate buffer (pH 4.5; KH_2PO_4+K_2HPO_4):methanol: acetonitrile in the proportion of 30:50:20 v/v. The mobile phase was set at a flow rate of 1.2 ml/min and the volume injected was 20µl for every injection. The detection wavelength was set at 271 nm.

Results: The developed method resulted in Metronidazole eluting at 2.92 min and Nalidixic acid at 3.99 min. Metronidazole exhibited linearity in the range 30-70µg/ml, while Nalidixic acid exhibited linearity in the range 45-105µg/ml. The precision is exemplified by relative standard deviations of 0.714% for Metronidazole and 0.398% for Nalidixic acid. Percentage Mean recoveries were found to be in the range of 98-102, during accuracy studies. The limit of detection (LOD) for Metronidazole and Nalidixic acid were found to be 2.48µg/ml and 2.24µg/ml respectively, while limit of quantitiation (LOQ) for Metronidazole and Nalidixic acid were found to be 7.51µg/ml and 6.79µg/ml respectively.

Conclusion: A simple, accurate, precise, linear and rapid RP-HPLC method was developed for simultaneous quantitative estimation of Metronidazole and Nalidixic acid in tablets and validated as per ICH guidelines. Hence it can be used for the routine analysis of Metronidazole and Nalidixic acid in tablets in various pharmaceutical industries.

Keywords: RP-HPLC, Metronidazole, Nalidixic acid, Method development, Validation.

INTRODUCTION

Metronidazole (fig. 1) chemically is 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl) ethanol [1]. Metronidazole is a nitro imidazole antibiotic drug used against anaerobic organisms, amoebozoa infections and antiprotozoal. It is frequently used for mild-to-moderate Clostridium difficile infection [2]. Metronidazole is also used to treat bacterial vaginosis, pelvic inflammatory disease, *pseudomembranous colitis*, *aspiration pneumonia*, *rosacea*, fungating wounds, intra-abdominal infections, lung abscess, gingivitis, amoebiasis, giardiasis, trichomoniasis, and infections caused by susceptible anaerobic organisms such as Bacteroides *fragilis spp*, *Fusobacterium spp*, *Clostridium spp, Peptostreptococcus spp* and *Prevotellaspp* [3]. It has a molecular formula of $C_6H_9N_3O_3$ and a molecular weight of 171.15 g/mol.

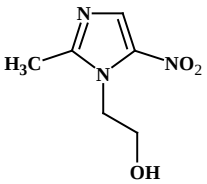

Fig. 1: Structure of Metronidazole

Nalidixic acid (fig. 2) chemically is 1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid. It is a synthetic quinolone antibiotic effective against both gram-positive and gram-negative bacteria. At lower concentrations, it acts as a bacteriostatic and at higher concentrations; it is bactericidal [4]. Nalidixic acid is an inhibitor of the A subunit of bacterial DNA gyrase. Nalidixic acid is indicated for the treatment of urinary tract infections caused by susceptible gram-negative microorganisms, including the majority of *E. Coli, Enterobacter* species, *Klebsiella* species, and *Proteus* species. It works by killing the bacteria and preventing their growth tract by stopping the production of essential proteins needed by the bacteria to survive. It has a molecular formula of $C_{12}H_{12}N_2O_3$ and a molecular weight of 232.235 g/mol.

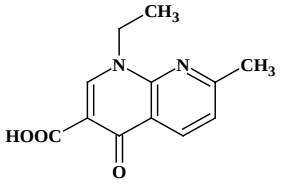

Fig. 2: Structure of Nalidixic acid

A detailed literature survey reveals that there exists literature on chromatographic methods for Nalidixic acid and Metronidazole individually and in combination with other drugs [4-24] in various matrices. There exist spectrophotometric [25], HPTLC [26] and supercritical fluid chromatographic method [27] for the simultaneous quantitative estimation of Metronidazole and Nalidixic acid in pharmaceutical dosage forms, while there is hardly any literature reported on RP-HPLC method development and validation for this combination. Hence we have explored in developing a new, accurate, precise, linear and a rapid isocratic RP-HPLC method for the simultaneous quantitative estimation of Metronidazole and Nalidixic acid in tablets and validate as per ICH guidelines.

MATERIALS AND METHODS

Chemicals and reagents

Analytically pure sample of Metronidazole and Nalidixic acid with purities greater than 99% were obtained as gift samples from Chandra Labs, Hyderabad, India and tablet formulation [Gramogyl Distab] was procured from Medplus pharmacy, Hyderabad, India

with labelled amount 100mg and 150mg of Metronidazole and Nalidixic acid respectively. Acetonitrile (HPLC grade) and Methanol (HPLC Grade) were obtained from Sigma Aldrich (Hyderabad, India) and SD Fine chem. (Hyderabad) respectively, water (HPLC grade), potassium dihydrogen ortho phosphate (KH_2PO_4) and di potassium hydrogen ortho phosphate (K_2HPO_4) (AR grade), ortho phosphoric acid (AR Grade) were obtained from SD Fine chemicals (Hyderabad, India), 0.45µm Nylon membrane filters were obtained from Spincotech Private Limited, Hyderabad, India.

Instrument

HPLC analysis was performed on Shimadzu LC-20AT VP Liquid Chromatograph comprising a LC-20AT pump, Shimadzu SPD-20A UV-VISIBLE detector and a reverse phase C18 column, Inertsil ODS 3V (250X4.6 mm; 5µ). A manually operating Rheodyne injector with 20 µL sample loop was equipped with the HPLC system. The HPLC system was controlled with "Spinchrom" software. A double beam UV-visible spectrophotometer Nicolet evolution 100 having two matched quartz cells with 1 cm light path was used for recording of spectra and measuring absorbance. An electronic analytical weighing balance (1mg sensitivity, Shimadzu BL220H), digital pH meter (Global digital) and sonicator (Citizen) were used in this study.

Methods

Selection of wavelength

Suitable wavelength for the HPLC analysis was determined by recording UV spectrums in the range of 200-400 nm for individual drug solutions of Nalidixic acid and Metronidazole. Suitable wavelength selected for simultaneous estimation is 271 nm (fig. 3-4).

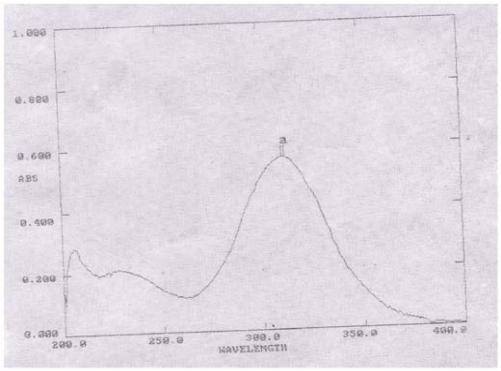

Fig. 3: UV spectrum of standard Metronidazole

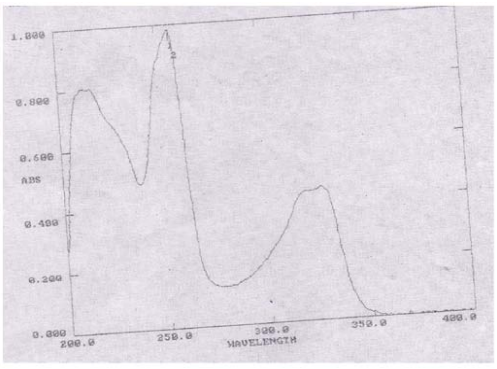

Fig. 4: UV spectrum of standard Nalidixic acid

Chromatographic conditions

The optimized method uses a reverse phase C18 column Inertsil ODS C18 column (250X4.6 mm×5µ) mobile phase consisting of mixed phosphate buffer (pH 4.5; KH_2PO_4+K_2HPO_4): methanol: acetonitrile in the proportion of 30:50:20 v/v. The mobile phase was set at a flow rate of 1.2 ml/min and the volume injected was 20µl for every injection. The detection wavelength was set at 271 nm.

Preparation of mixed buffer solution

1.625 gm of potassium dihydrogen orth phosphate (KH_2PO_4) and 0.3 gm of dipotassium hydrogen ortho phosphate (K_2HPO_4) was weighed and dissolved in 100 ml of water and volume was made up to 1000 ml with water. Adjust the pH to 4.5 using ortho phosphoric acid and potassium hydroxide solution. The buffer was filtered through 0.45µ filters to remove all fine particles and gases.

Mobile phase preparation

The mobile phase was prepared by mixing acetonitrile, methanol and mixed phosphate buffer in the ratio of 20:50:30 v/v and later it was sonicated for 10 minutes for the removal of air bubbles.

Diluent

Diluent used is the mobile phase itself.

Preparation of mixed standard solution

Weigh accurately 75mg of Nalidixic acid and 50 mg of Metronidazole in 100 ml of volumetric flask and dissolve in 10 ml of mobile phase and make up the volume with mobile phase. From above stock solution 75µg/ml of Nalidixic acid and 50µg/ml of Metronidazole is prepared by diluting 1 ml to 10 ml with mobile phase. This is treated as mixed working standards solution, 100% target concentration.

Preparation of stock and working sample solution

10 tablets were weighed and taken into a mortar, crushed and then uniformly mixed. Test stock solutions of Nalidixic acid (1500µg/ml) and Metronidazole (1000µg/ml) were prepared by dissolving average of 10 tablets, equivalent to 150mg of Nalidixic acid and 100mg of Metronidazole and made up to 100 ml with mobile phase. Sonicated for 5 min and later filtered the solution using 0.45micron syringe filter. 0.5 ml of the above stock solution was pipetted out and made up to 10 ml to get working sample solution equivalent to a concentration of 75µg/ml for Nalidixic acid and 50µg/ml for Metronidazole, concentrations equal to 100% target concentration.

RESULTS AND DISCUSSION

Method development

A reverse phase HPLC method was developed keeping in mind the system suitability parameters i. e. Resolution factor (Rs) between peaks, Asymmetric factor (A), number of theoretical plates (N), runtime and the cost effectiveness. The optimized method developed resulted in the elution of Nalidixic acid at 3.99 min and Metronidazole at 2.92 min. Fig. **5** and **6** represent chromatograms of blank solution and mixture of standard solutions respectively. The total run time is 6 minutes.

System suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (*Rt*), number of theoretical plates (*N*), peak resolution (Rs) and Asymmetric factor (A) was evaluated for six replicate injections of the standards at working concentration. The results given in table 1 were within acceptable limits.

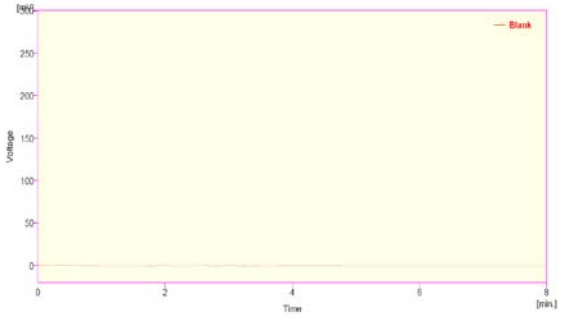

Fig. 5: Typical chromatogram of blank solution

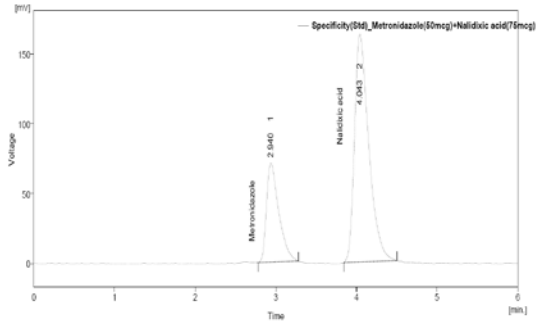

Fig. 6: Typical chromatogram of mixture of standards solution

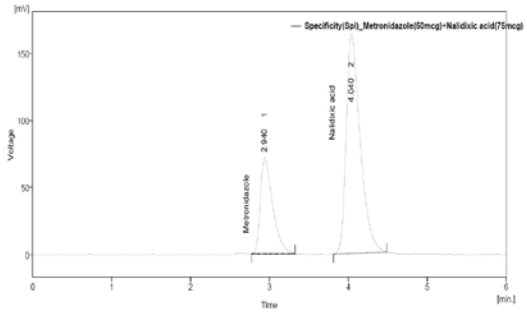

Fig. 7: Typical chromatogram of sample solution

Table 1: System suitability studies results

Parameters	Acceptance Limits	Nalidixic acid	Metronidazole
Retention time (min)	-	3.994	2.921
Resolution factor (Rs)	Not less Than 2	3.577	
Number Of Theoretical plates (N)	Not less Than 2000	4492	3749
Tailing factor (T)	Not More Than 2	1.9	1.41

In order to test the applicability of the developed method to a commercial formulation, 'Gramogyl Distab' tablets were chromatographed at working concentration and it is shown in fig. 7. The sample peaks were identified by comparing the relative retention times with the mixture of standards solution (fig. 6-7). System suitability parameters were within the acceptance limits, ideal for the chromatographed sample. Integration of separated peak area was done and each drug concentration was determined by using the peak area concentration relationship obtained in the standardization step. The protocol affords reproducible quantification of the two drugs with error less than 10%, which is the standard level in any pharmaceutical quality control.

Method validation

Validation of the analytical method is the process that establishes by laboratory studies in which the performance characteristics of the method meet the requirements for the intended analytical application. HPLC method developed was validated according to International Conference on Harmonization (ICH) guidelines [28] for validation of analytical procedures. The method was validated for the parameters like linearity, accuracy, system precision, intra-day precision, limit of detection (LOD) and limit of quantitiation (LOQ).

Specificity

Fig. 5-7 for blank, mixture of standards drug solution and sample chromatogram reveal that the peaks obtained in the standards solution and sample solution at working concentrations are only because of the drugs as blank has no peak at the retention time of Nalidixic acid and Metronidazole standards. Accordingly it can be concluded that, the method developed is said to be specific.

Precision

System precision

Six replicate injections of the mixture of standards solution at working concentration showed % RSD (Relative Standard Deviation) less than 2 concerning peak area for both the drugs, which indicate the acceptable reproducibility and thereby the precision of the system. System precision results are tabulated in Tables 2-3.

Method precision

Method precision was determined by performing assay of the sample under the test of repeatability (Intraday precision) at working concentrations.

Repeatability (Intraday precision)

Six consecutive injections of the sample from the same homogeneous mixture at working concentration showed % RSD less than 2 concerning % assay for both the drugs which indicate that the method developed is method precise by the test of repeatability and hence can be understood that the method gives consistently reproducible results (Table 4).

Table 2: System precision results of Nalidixic acid

Injection	Retention time (min)	Peak area
1	3.993	2012.58
2	3.993	2009.765
3	3.990	2023.634
4	3.987	2028.946
5	3.990	2031.51
6	4.010	2035.765
Mean	3.994	2023.700
SD	0.008	10.506
%RSD	0.21	0.52

Table 3: System precision results of Metronidazole

Injection	Retention time (min)	Peak area
1	2.917	765.269
2	2.920	760.464
3	2.923	768.530
4	2.923	763.825
5	2.917	762.172
6	2.930	775.496
Mean	2.921	765.959
SD	0.0049	5.425
%RSD	0.17	0.71

Table 4: Intraday precision results of Metronidazole and Nalidixic acid

Injection	% Assay (Nalidixic acid)	% Assay (Metronidazole)
1	100.05	99.08
2	99.75	100.61
3	99.59	99.28
4	100.75	99.5
5	99.94	98.43
6	100.02	99.38
Mean	100.022	99.38
SD	0.399	0.71
%RSD	0.3989	0.714

Linearity

Standards solutions of Metronidazole and Nalidixic acid at different concentrations were prepared. Calibration curves (fig. 8-9) were constructed by plotting the concentration level versus corresponding peak area for both the drugs. The results show an excellent correlation between peak areas and concentration within the concentration range of 45-105µg/ml for Nalidixic acid and 30-70µg/ml for Metronidazole (Tables 5-6). The correlation coefficients were greater than 0.99 for both the drugs, which meet the method validation acceptance criteria and hence the method is said to be linear for both the drugs.

Table 5: Calibration data for Nalidixic acid

Concentration (µg/ml)	Peak Area
45	1131.154
60	1589.595
75	2210.885
90	2728.440
105	3181.713
Regression equation	y=34.93x-451.6
Regression coefficient	0.997

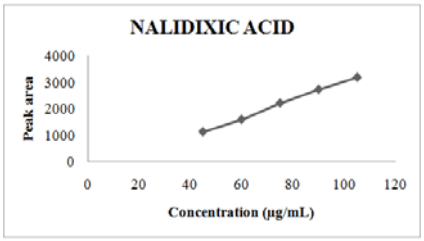

Fig. 8: Linearity graph of Nalidixic acid

Table 6: Calibration data for Metronidazole

Concentration (µg/ml)	Peak Area
30	455.279
40	637.105
50	898.173
60	1062.233
70	1295.814
Regression equation	y=21.06x-183.3
Regression coefficient	0.996

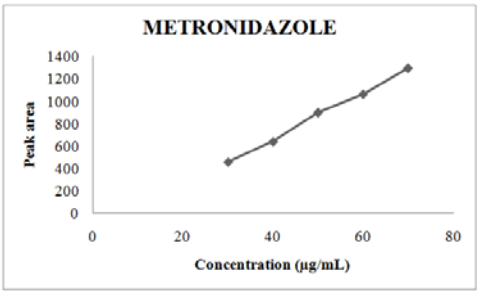

Fig. 9: Linearity graph of Metronidazole

Accuracy

Accuracy was determined by means of recovery experiments, by the determination of % mean recovery of both the drugs at three different levels (80-120%). At each level, three determinations were performed. Percent mean recovery is calculated as showed in table 7**.** The accepted limits of mean recovery are 98% -102% and all observed data were within the required range, which indicates good recovery values and hence the accuracy of the method developed.

Table 7: Results of Accuracy studies for Metronidazole and Nalidixic acid

Concentration level (%)	% Mean recovery metronidazole	% Mean recovery nalidixic acid
80	98.20	101.43
100	98.11	101.96
120	100.47	99.75

Sensitivity

The sensitivity of measurement of Metronidazole and Nalidixic acid by use of the proposed method was estimated in terms of the limit of quantitation (LOQ) and limit of detection (LOD). LOQ and LOD were calculated by the use of the equations LOD = 3.3σ/S and LOQ = 10σ/S where σ is the standard deviation of response of calibration plots and S is the slope of the corresponding calibration plot. The limit of detection (LOD) for Metronidazole and Nalidixic acid were found to be 2.48µg/ml and 2.24µg/ml respectively, while limit of quantitiation (LOQ) for Metronidazole and Nalidixic acid were found to be 7.51µg/ml and 6.79µg/ml respectively.

CONCLUSION

A reverse phase HPLC isocratic method developed has been validated as per ICH guidelines in terms of specificity, accuracy, precision, linearity, limit of detection and limit of quantitation, for for simultaneous quantitative estimation of Metronidazole and Nalidixic acid in tablets. The developed method resulted in Metronidazole eluting at 2.92 min and Nalidixic acid at 3.99 min. Metronidazole exhibited linearity in the range 30-70µg/ml, while Nalidixic acid exhibited linearity in the range 45-105µg/ml. The precision is exemplified by relative standard deviations of 0.714% for Metronidazole and 0.398% for Nalidixic acid. Percentage Mean recoveries were found to be in the range of 98-102, during accuracy studies. The limit of detection (LOD) for Metronidazole and Nalidixic acid were found to be 2.48µg/ml and 2.24µg/ml respectively, while limit of quantitiation (LOQ) for Metronidazole and Nalidixic acid were found to be 7.51µg/ml and 6.79µg/ml respectively.

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CONFLICT OF INTERESTS

Declared None

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