Research Article

RP-HPLC Method for the Simultaneous Determination of Metronidazole, Tinidazole, Ornidazole, Secnidazole and Ofloxacin in Bulk and Pharmaceutical Dosage Form

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ABSTRACT

An RP-HPLC method for the simultaneous determination of Metronidazole, Tinidazole, Ornidazole, Secnidazole, and Ofloxacin in tablets was developed and validated as per ICH & FDA guidelines. The separation was achieved with a 150 mm x 3.0 mm, 3.5 µm C18 column, by using a simple linear gradient. Mobile phase A was Buffer (0.2% Triethylamine containing 20mM phosphate buffer pH adjusted to 7.0 with Orthophosphoric acid) and Mobile Phase B was, mixture of Acetonitrile and methanol in the ratio 15:85 v/v with simple gradient program was delivered at a flow rate of 0.5 mL/min. The column temperature was kept at 30°C. The detector was set at the wavelength of 290 nm and 320 nm. Injection volume kept was 10 µL. The gradient separation was achieved within 20 minutes. The linearity of the proposed method was investigated in the range 0.100-0.300 mg/mL (r2= 1.000) for Metronidazole, 0.125-0.375 mg/mL (r2= 1.000) for Tinidazole, 0.125-0.375 mg/mL (r2= 1.000) for Ornidazole, 0.250-0.725 mg/mL (r2= 1.000) for Secnidazole, and 0.050-0.150 mg/mL (r2= 1.000) for Ofloxacin. The assay method is considered to be specific as there was no blank and placebo interference at retention time of Metronidazole, Tinidazole, Ornidazole, Secnidazole, and Ofloxacin peaks. The developed method has an advantage that all the drugs can be quantified alone or in combination using a single mobile phase.

Keywords: RP-HPLC, ICH, Validation, Metronidazole, Ornidazole, Tinidazole, Secnidazole, Ofloxacin.

INTRODUCTION

Many antifungal drugs like nitroimidazole contain an imidazole ring.[1-5] From the chemistry perspective, nitroimidazole antibiotics can be classified according to the location of the nitro functional group. Nitroimidazole antibiotics have been used to combat anaerobic bacterial and parasitic infections.[2,4-9] Metronidazole, Tinidazole, Ornidazole, Secnidazole, and Ofloxacin are members of nitroimidazole class of drugs.[1]

Metronidazole, (2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanol) is used particularly for anaerobic bacteria and protozoa. Tinidazole, (1-[2-(ethanesulfonyl)ethyl]-2-methyl-5-nitro-1H-imidazole) is a synthetic antiprotozoal agent. Tinidazole is substituted benzimidazole and chemically known as 5-methoxy-2-[[(4-methoxy-3, 5-dimethyl-2-pyridinyl) methyl] sulfinyl]-1H-benzimidazole. Secnidazole[10-12], (1-(2-hydroxypropyl)-2-methyl-5-nitroimidazole, is anti-infective used in the treatment of dientamoebiasis. Ornidazole, (1-chloro-3-(2-methyl-5-nitro-1H-imidazol-1-yl)propan-2-ol), is an antiamoebic agent that is given to patients with amoebic dysentery. Ofloxacin, as (8-Fluoro-3-methyl-9-(4-methyl-piperazin-1-yl)-6-oxo-2,3-dihydro-6H-1-oxa-3a-aza-phenalene-5-carboxylic acid), is a synthetic antibiotic of the fluoroquinolone drug class considered to be a second-generation fluoroquinolone.

Literature survey revealed that there are only few methods available to detect or analyse residue of one or more nitroimidazoles. For several of these methods, the use of gas[12] or liquid[13–19] chromatography coupled with a mass spectrometer, gives them their principal advantage as techniques of confirmation and identification. However, none of the method available has been reported for the simultaneous determination of Metronidazole, Tinidazole[17], Secnidazole[14,15,17], Ornidazole and Ofloxacin in pharmaceutical dosage form with great difference in label claim (Metronidazole 400 mg, Tinidazole 500 mg, Ornidazole 500 mg, Secnidazole 1000 mg and Ofloxacin 200 mg).

The method was validated as per the present ICH guideline on validation of analytical procedure Q2A (R1).[18,19]

Quantitation was achieved with UV detection at 285 nm and 320 nm based on peak area with linear calibration curves at different concentration ranges. The method was linear over wide concentration range of 0.100-0.300 mg/ml for Metronidazole, 0.125-0.375 mg/ml for Tinidazole, 0.250- 0.725 mg/ml for Secnidazole, 0.050-0.150 mg/ml for Ornidazole and 0.050-0.150 mg/ml for Ofloxacin. The accuracy of the method was evaluated in triplicate at three concentration level i.e. 80%, 100% and 120% of target test concentration.

MATERIALS AND METHODS

Chemicals and Reagents

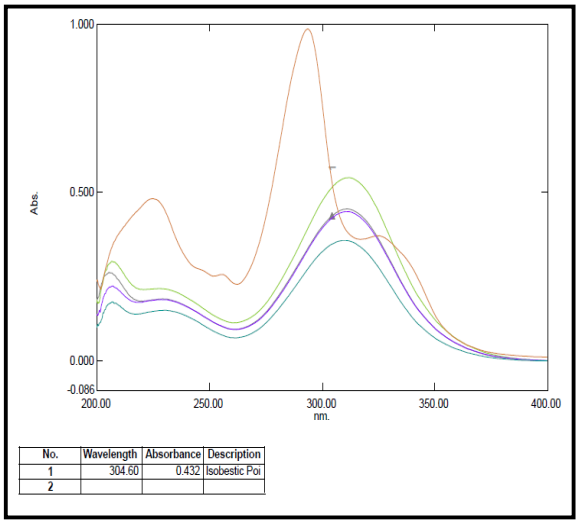
Ornidazole (ONZ), Tinidazole (TNZ), Secnidazole (SCZ), Ofloxacin (OFLOX) and Metronidazole (MTZ) were obtained from Bioleo Labs and K.P Labs, Hyderabad, India. Excipients were obtained from K.P. LABS, Hyderabad, India. Branded formulation of Secnidazole,

Metronidazole, Ornidazole, Tinidazole, and Ofloxacin were procured from local market.

HPLC grade methanol, acetonitrile, triethylamine and Orthophosphoric acid (88%) were from Merck (Mumbai, India). HPLC grade water was prepared using a Milli-Q system (Millipore). Nylon syringe filters (0.45 µm) were from Millipore (Mumbai, India). All reagents used were of analytical grade.

Selection of UV wavelength

10 ppm solution of each Secnidazole, Metronidazole, Tinidazole, Ornidazole and Ofloxacin were prepared separately in methanol. UV scan of the above solutions were carried out over a wavelength range of 200–400 nm by using the Shimadzu UV spectrophotometer, Model-UV-1800. The detection wavelength was set at 290 nm and 320 nm because components had higher responses. An overlaid UV absorption spectrum is shown in Figure-1.



No.	Wavelength	Absorbance	Description
1	304.60	0.432	Isobestic Poi
2			

Figure 1: Overlaid UV absorption spectrum of Secnidazole, Metronidazole, Tinidazole, Ornidazole and Ofloxacin.

HPLC instruments and analytical conditions

Chromatographic separation was achieved using HPLC System (Waters Alliance 2695 Separation Module) containing binary solvent manager, an autosampler and PDA detector. The output signal was monitored and processed using Empower software.

Waters X Bridge C18 column (150 mm X 3.0 mm id and 3.5 µm particle size) was used as the stationary phase. Mobile phase consisting of Mobile phase A: Buffer (0.2% Triethylamine containing 20 mM Potassium dihydrogen orthophosphate, pH was adjusted to 7.0 with orthophosphoric acid) and Mobile Phase B, Acetonitrile and methanol in the ratio 15:85 v/v with simple gradient program (0-3 min :: MP-A : 85-85; 3-7 min :: MP-A : 85-80; 7-14min :: MP-A : 80-20; 14-15 min :: MP-A : 20-85; 15-20 min :: MP-A : 85-85) was delivered at a flow rate of 0.5 mL/min. The column temperature was kept at 30°C. The detector was set at the wavelength of 290 nm and 320 nm. Injection volume was kept 10 µL.

Solutions and sample preparation

For the system suitability test, the solution contains Metronidazole (0.20 mg/mL), Tinidazole (0.25 mg/mL), Secnidazole (0.50 mg/mL), Ornidazole (0.25 mg/mL) and Ofloxacin (0.10 mg/mL).

For the linearity studies, variable weight of compounds was weighed and diluted with the solvent to yield solutions at different concentration.

For test sample solution, 5 tablets were weighed and transferred to 250 mL volumetric flask. Added 100 mL of solvent mixture (Water and Methanol; 1:1 v/v) and 0.5 ml of 1N HCl to this mixture. Sonicated and vortex for about 10 minutes. Cooled to room temperature and diluted to the volume with diluent.

Further 2.5 mL aliquot of this sample stock solution was transferred to 100 mL volumetric flask and diluted to the mark with diluent to obtain a test solution of Secnidazole (0.50 mg/mL), Ornidazole (0.25 mg/mL), Tinidazole (0.25 mg/mL), Metronidazole (0.20 mg/mL) and Ofloxacin (0.10 mg/mL). The solution was filtered through Nylon 0.45 µm membrane filter.

10 µL of these solutions were injected and the peak area was recorded from the respective chromatogram.

Calculation

All active ingredients were quantified with the following calculation:

$$\%\ Assay\ = \frac{Sample\ Area \times Standard\ dilution\ factor}{Standard\ Area \times Sample\ dilution\ factor} \times 100$$

RESULTS AND DISCUSION

Literature survey revealed that, no HPLC method is available in the official compendia for simultaneous determination of Secnidazole, Metronidazole, Tinidazole, Ornidazole and Ofloxacin in bulk and in dosage forms. The present proposed method was compared with the reported method in the literature and comparison is shown in Table-1. The complete separation of the analytes was accomplished in less than 20 min and the method can be successfully applicable to perform routine analysis of Secnidazole, Metronidazole, Tinidazole, Ornidazole and Ofloxacin in bulk and in commercially available dosage forms.

Method Validation

The developed RP-HPLC method was validated as per International Conference on Harmonization (ICH) guideline, VALIDATION OF ANALYTICAL PROCEDURES: Q2 (R1)[18], for the parameters like system suitability, linearity and range, precision (repeatability), intermediate precision (ruggedness), specificity, accuracy and robustness.

System suitability

The system suitability test performed according to USP37.[19] The standard solution was injected six times and

results were recorded to find adequate peak separation (resolution), percentage relative standard deviation for area and retention time, peak asymmetry and theoretical plates. The results obtained were compiled in Table-2.

Table 1: Comparison of the performance characteristics of the present method with the published methods

S. No.	Method	Reagents	Detection Wavelength/Runtime	Linearity (mg/mL)	Remark	Reference
1	Spectro photometry	0.5% Sulphanilamide 0.3% NEDA	540 nm (Coloured complex)	Not applicable	Direct spectrophoto-metric method was used which have limited sensitivity.	[36]
2	HPLC	Triethylamine, Potassium dihydrogen orthophosphate, Acetonitrile, Methanol, Orthophosphoric acid.	290 nm and 320 nm / 20 min	0.100-0.300 mg/ml for Metronidazole, 0.125-0.375 mg/ml for Tinidazole, 0.250- 0.725 mg/ml for Secnidazole, 0.125-0.375 mg/ml for Ornidazole and 0.050-0.150 mg/ml for Ofloxacin.	Wide linearity range and also study was performed in combined form.	Present work

Table 2: System suitability

Reference solution Peak Area, for n=6					
	Metronidazole	**Tinidazole**	**Secnidazole**	**Ornidazole**	**Ofloxacin**
%RSD	0.33	0.34	0.60	0.38	0.29
Acceptance Criteria	Not more than 2.0%				
Reference solution Peak resolution, for n=6					
Resolution	-	4.8	3.6	9.0	6.8
Acceptance Criteria	Not less than 2.0				
Reference solution Peak Symmetry factor, for n=6					
Symmetry Factor	1.20	1.15	1.21	1.2	1.4
Acceptance Criteria	Should be between 0.8 – 1.5				
Reference solution Peak Theoretical plates, for n=6					
Theoretical plates	2100	2860	5040	18662	33490
Acceptance Criteria	Not less than 1500				

Results: It was observed that limits for percentage standard deviation for peak areas, symmetry factor and theoretical plates for all individual analytes were within the limit, which shows that the method has good system suitability.

Table 3: Precision and Intermediate Precision results

	Metronidazole	**Tinidazole**	**Secnidazole**	**Ornidazole**	**Ofloxacin**
Precision (Day 1) –Assay %					
Average Assay (%)	100.31	100.15	99.72	100.23	100.02
%RSD	**0.37**	**0.42**	**0.61**	**0.58**	**0.28**
Intermediate Precision (Day 2) – Assay %					
Average	99.90	100.03	99.74	99.82	100.36
%RSD	**0.50**	**0.46**	**0.43**	**0.46**	**0.19**
Average for Precision and Intermediate Precision	100.11	100.09	99.73	100.03	100.19
% RSD for Precision and Intermediate Precision	**0.29**	**0.08**	**0.01**	**0.29**	**0.24**
Acceptance Criteria	**%RSD should not be more than 2.0% for day-1 and day-2.**				

Results: %RSD obtained was found to be less than 2% for day – 1 and day -2

Table 4: Accuracy (Recovery)

Active Ingredient Name	Concentration (%)	Amount Added (mg/mL)	Amount found (mg/mL)*	Mean Recovery (%)**	Average Recovery (%)
Metronidazole	80	0.16028	0.16029	100.01	99.90
	100	0.19965	0.19908	99.71	
	120	0.23947	0.23943	99.99	
Tinidazole	80	0.19898	0.19938	100.20	100.03
	100	0.24916	0.24868	99.81	
	120	0.30007	0.30028	100.07	
Secnidazole	80	0.40110	0.39991	99.70	99.74
	100	0.49420	0.49424	100.01	
	120	0.59274	0.58981	99.50	
Ornidazole	80	0.20058	0.19979	99.61	99.82
	100	0.24930	0.24842	99.64	
	120	0.29843	0.29903	100.20	
Ofloxacin	80	0.8165	0.8203	100.47	100.36
	100	0.10105	0.10123	100.18	
	120	0.12002	0.12053	100.42	
Acceptance criteria	The mean and individual recoveries should be within 98.0 – 102.0%				

* mean of 3 readings for individual level; ** Average recovery for all levels

Results: Accuracy results obtained shows that the mean and individual recoveries were in range of 98.0 – 102.0%

Table 5: Robustness results

Summary of system suitability Parameters															
Variations	Resolution					Symmetry Factor					Theoretical plates				
	METRONIDAZOLE	TINIDAZOLE	SECNIDAZOLE	ORNIDAZOLE	OFLOXACIN	METRONIDAZOLE	TINIDAZOLE	SECNIDAZOLE	ORNIDAZOLE	OFLOXACIN	METRONIDAZOLE	TINIDAZOLE	SECNIDAZOLE	ORNIDAZOLE	OFLOXACIN
0.5 mL/min 30°C	-	4.57	3.58	8.66	7.78	1.19	1.15	1.22	1.14	1.44	2067	2803	4691	13631	35084
0.4 mL/min	-	5.71	3.97	10.77	8.93	1.32	1.20	1.22	1.06	1.16	2500	3533	6716	35296	94863
0.6 mL/min	-	4.59	3.70	8.72	10.41	1.35	1.28	1.28	1.12	1.13	2073	2769	4614	12364	91484
25°C	-	4.40	3.72	8.85	7.81	1.26	1.39	1.25	1.31	1.41	2091	2361	3429	8354	25223
35°C	-	3.34	2.72	5.85	4.81	1.45	1.49	1.45	1.41	1.48	2091	2161	3029	8354	19223
Buffer pH=6.8	-	4.17	2.97	7.30	6.93	1.36	1.35	1.43	1.32	1.29	2071	2024	3148	11133	54978
Buffer pH=7.2	-	3.21	2.24	8.73	7.92	1.30	1.21	1.23	1.09	1.09	2062	2648	4462	16209	76162
Acceptance Criteria		Not less than 2.0					Not more than 1.5					Not less than 2000			

Results: From variation in Temperature, flow rate and Buffer pH variation, it was observed that there were no marked changes in the chromatograms, which demonstrated that the method developed is robust. Resolution, symmetry factor and Theoretical plate limits for flow rate variation and temperature variation were within the acceptance criteria, which show that the method exhibits a good system suitability under given set of conditions.

Table 6: Solution Stability results

Test Solution - Solution stability										
Time (Hours)	% Assay of MTZ	% Change w.r.t. Initial	% Assay of TNZ	% Change w.r.t. Initial	% Assay of SCZ	% Change w.r.t. Initial	% Assay of ONZ	% Change w.r.t. Initial	% Assay of OFLOX	% Change w.r.t. Initial
Initial	99.36	N/A	99.07	N/A	99.15	N/A	99.15	N/A	99.95	N/A
10	99.48	0.12	99.17	0.11	99.94	0.79	99.24	0.10	99.97	0.02
18	99.84	0.48	99.42	0.36	99.81	0.66	99.24	0.09	99.88	0.07
30	99.60	0.24	99.27	0.21	99.67	0.52	99.23	0.08	99.78	0.17
Acceptance Criteria :	% Change w.r.t. initial for Test solution should be NMT 1.0% of initial assay results.									
Reference Solution - Solution stability										
Time (Hours)	Area of MTZ	% Change w.r.t. Initial	Area of TNZ	% Change w.r.t. Initial	Area of SCZ	% Change w.r.t. Initial	Area of ONZ	% Change w.r.t. Initial	Area of OFLOX	% Change w.r.t. Initial
Initial	11703907	N/A	9890378	N/A	24960296	N/A	11022041	N/A	8236900	N/A
10	11898024	0.46	9989197	0.14	25363329	0.17	11247578	0.41	8319562	0.15
18	11890774	0.40	10063070	0.27	25417277	0.22	11236831	0.31	8352131	0.54
30	11899384	0.47	10111065	0.21	25456912	0.38	11252322	0.45	8365310	0.70
Acceptance Criteria :	% Change w.r.t. initial for reference solution should NMT 1.0% of initial.									

Results: Both Test and reference solution was found to be stable upto 30 hours, at 25 °C (laboratory temperature).

Specificity

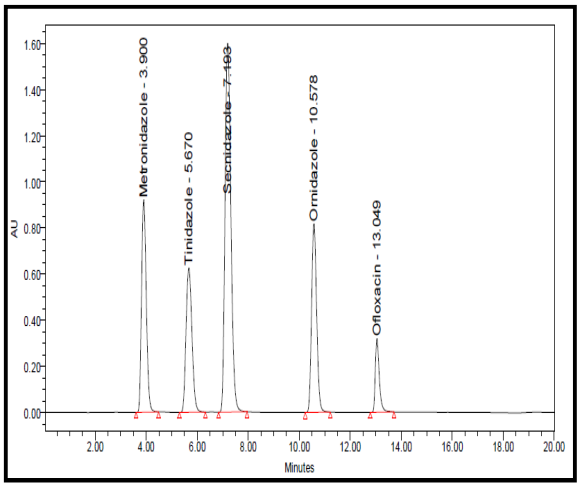


Figure 2: Typical Chromatograms of Standard Solution containing Metronidazole, Tinidazole, Ornidazole, Secnidazole and Ofloxacin

Specificity was performed to detect the presence of interference peak (blank and placebo) at the retention time of analyte peak. The specificity of the method was checked by comparison of chromatograms obtained from test sample solution and the corresponding placebo. The interference of placebo was detected by preparing placebo solution equivalent to about the weight in proportion of tablet preparation as per test method and was injected into the HPLC system. The interference of blank was detected by injecting diluent as per test method.

The representative chromatogram obtained for Secnidazole, Metronidazole, Tinidazole, Ornidazole and Ofloxacin is shown in Figure-2.

Results: No interference from diluent, excipients or any other peak was found at retention time of Metronidazole, Tinidazole, Ornidazole, Secnidazole and Ofloxacin.

Precision and Ruggedness (Intermediate precision)

Method precision was evaluated by carrying out six different test sample solution preparation. Different analysts from the different laboratory evaluated the intermediate precision of the method.

Assay of these samples were determined. Precision and intermediate precision of the method was evaluated by calculating the %RSD. The values are given in Table-3.

Linearity and range

Linearity of detector response was determined by preparing a series of solution of working standards (mixture of all active ingredients) over the range of 80% to 120% of targeted concentration. These solutions were injected and response area was recorded. Calibration curve was constructed by plotting area against concentration and regression equation was computed. The linearity plots with values are shown in Figure-3.

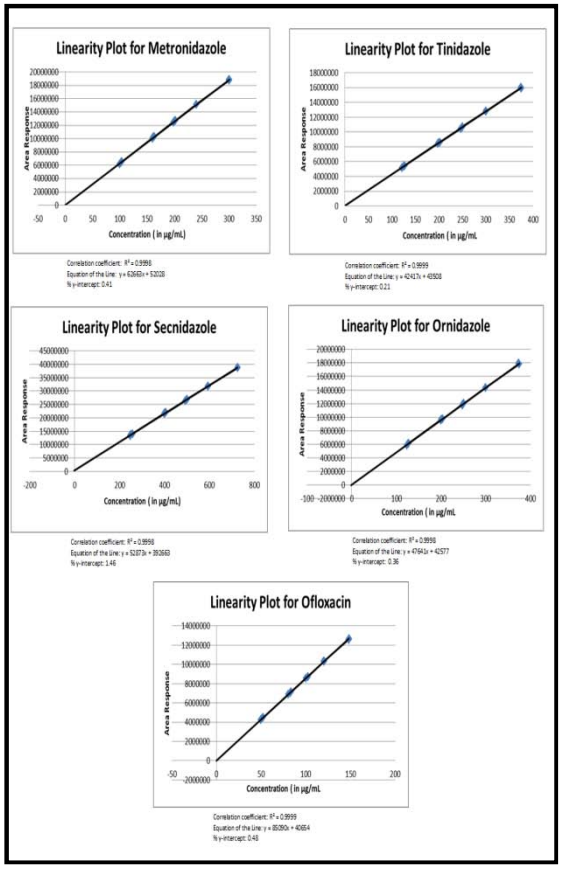


Figure 3: Calibration curves showing linearity

Results: The correlation coefficient values were within the limit 0.998 and Y-intercept values were within ± 2 %.

Accuracy (Recovery)

To study the accuracy of the method recovery experiments were carried out. The accuracy of the test method was determined by varying weights of crushed test sample at the level of 80%, 100% and 120% of targeted concentration. The recovery samples were prepared in triplicate at each level. The samples at different levels were chromatographed and the percentage recovery for the amount added was calculated. The values were given in Table-4.

Robustness - Effect of variation in Temperature and variation in flow rate

To study robustness of test method, small, deliberate changes were made to the chromatographic condition. A study was performed by changing the temperature, buffer pH and flow rate. Standard solution prepared as per test method and injected into the HPLC system at 25°C temperature. Flow rate change was done by varying flow rate at from 0.5 mL/min to 0.4 mL/min and 0.6 mL/min.

System suitability parameters were evaluated. The values are given in Table-5.

Solution Stability

To assess the solution stability, reference standard and test solutions were kept at 25 °C (laboratory temperature) for 24 hours, and injected in HPLC system at predetermined time interval.

The percentage change with respect to initial of test and reference standard solutions were evaluated. The values were given in Table-6.

CONCLUSION

A gradient RP-HPLC method has been developed and validated for the analysis of Secnidazole, Metronidazole, Tinidazole, Ornidazole and Ofloxacin by RP-HPLC in tablet dosage forms. The results of the method validation revealed that the assay method is specific, selective, linear, accurate and robust. The validation performed further gives documented evidence, that the analytical method for the simultaneous estimation of Secnidazole, Metronidazole, Tinidazole, Ornidazole and Ofloxacin by RP-HPLC in tablet dosage forms will consistently analyze these drugs quantitatively in combination and single dosage form and can be used for routine analysis in quality control and R&D laboratory.

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