Original Article

Stability Indicating Assay Method Development and Validation for Simultaneous Estimation of Ofloxacin and Ornidazole by RP-HPLC in Bulk: An Application to Tablet Formulation and Dissolution Studies

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ABSTRACT

Aim: The present article focuses on development of sensitive, simple, precise, accurate and inexpensive stability indicating assay method for the simultaneous estimation of Ofloxacin and Ornidazole in bulk was established using RP-HPLC. **Materials and Methods:** The separation was done with C_{18} Phenomenex Hyperclone BDS column (250 × 4.6mm, 5μ) at a temperature of 25°C using a mobile phase of acetonitrile: pH 5.8 ammonium acetate buffer of ratio 25:75 with a flow rate of 1ml/min. The detection was done at 293nm and 311nm and the retention time for Ofloxacin and Ornidazole was 4.278 min and 6.750 m respectively. **Results:** The method was seen to be linear over the range of 2-16μg/ml for both drugs. The method was precise and robust with LOD of 0.331 and 0.360 and LOQ of 1.005 μg/ml and 1.092 μg/ml for Ofloxacin and Ornidazole respectively. The drugs were subjected to stress conditions in acidic, alkaline, oxidative, thermal and photolytic conditions. **Conclusion:** The method for this simultaneous estimation was found to be accurate, precise, fast and simple with a run time within 8 min. This method developed was applied with success for the assay and dissolution studies in tablet formulation.

Key words: Ofloxacin, Ornidazole, Stability indicating method, HPLC, Dissolution.

INTRODUCTION

Ofloxacin(7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1azatricyclo[7.3.1.05,13] trideca-5(13),6,8,11-tetraene-11-carboxylic acid; hydrochloride) falls under a class of antibiotics named Quinolone antibiotics (Figure 1). It is deemed to be very versatile in terms of infections originating from bacteria mainly infections of the skin, lungs, bladder and kidneys, or prostrate by impairing the bacterial growth by suppressing the enzymes mostly responsible for actions such as of transcription, repair, recombination and replication of DNA gyrase and bacterial topoisomerase IV (belonging to the class of type II topoisomerases). It is reported officially in the United States Pharmacopoeia, British Pharmacopoeia and Indian Pharmacopoeia. Ornidazole (1-chloro-3-(2-methyl-5-nitroimidazol-1-yl)propan-2-ol) has been derived from 5-nitroimidazole (Figure 1) and is effective in attacking anaerobic bacteria and protozoa. This is transformed into reduction products that bind with DNA causing degradation of both the framework and strand of helical DNA, leading to protein synthesis inhibition and apoptosis in vulnerable organisms. It is official in Indian Pharmacopoeia. B. Dhandapani and N. Thirumoorthy 2010.[1] Developed a RP-HPLC method to quantify ofloxacin and ornidazole in tablet formulation. Nooman A. Khalaf, Ashok K. Shakya and Maher Shurabji 2010.[2] Developed a RP-HPLC method to quantify ofloxacin

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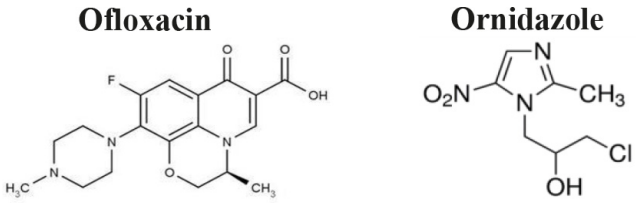

Figure 1: Structures of Ofloxacin and Ornidazole.

and ornidzole in tablet formulation. Shaik Mohammed Yusuf, E. Vijay Kumar, C. Surya Svarny and E. Divya.[3] Developed a new RP-HPLC method for the simultaneous estimation of Ornidazole and Ofloxacin in its bulk and tablet dosage form. Bhusari KD and Chaple DR 2009.[4] Developed a spectrophotometric method for the simultaneous estimation in tablet dosage form of ofloxacin and ornidazole. Saumil Mehta and Sukhdev Singh.[5] Developed a spectrophotometric method for the simultaneous estimation in combined dosage form of ofloxacin and ornidazole using UV spectrophotometer. The Indian Pharmacopoeia, Volume III 2018.6 was referred for the dissolution procedure.

Literature study showed that analytical techniques *viz.* HPTLC and HPLC methods have been presented for simultaneous determination of ofloxacin and ornidazole. There is no article related to Stability Indicating HPLC method to quantify Ofloxacin and Ornidazole in bulk has ever been mentioned within literature referred. The primary goal of this study was to produce a specific, accurate and reproducible stability indicating HPLC method for estimation of Ofloxacin (OFL) and Ornidazole (ORN) as stated in the ICH guidelines.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals were of analytical grade. Ofloxacin and Ornidazole working standards were obtained as gift samples from AKUMS Drugs and Pharmaceuticals Ltd (Haridwar, India). Methanol (HPLC grade) and potassium hydroxide (analytical grade) were bought from Finar Limited (Mumbai, India), triethylamine (HPLC grade) and acetonitrile (HPLC grade) were bought from Spectrochem Pvt. Ltd. (Mumbai, India) and potassium dihydrogen phosphate (analytical grade) and ammonium acetate (analytical grade) was bought from Lobachem (Mumbai, India). The tablet formulation was (Oflox Oz, Cipla Ltd., Sikkim, India) containing 200mg of ofloxacin and 500mg of ornidazole was obtained locally and thus used for the analysis of the marketed formulation.

Instrumentation

The liquid chromatographic system used was Shimadzu LC20 AD (Shimadzu, Kyoto, Japan) equipped with gradient pump, auto- injector, photo diode array detector and also UV-Vis detector. LC solution software on a C_{18} Phenomenex Monomeric 130A (250 × 4.6mm, 5µm) was utilised to carry out the chromatographic analysis. Dissolution system used was Electrolab dissolution tester (USP) TDT-08L.

Preparation of Buffer

10mM ammonium acetate buffer (pH 5.8± 0.02): 0.77 g of ammonium acetate was dissolved in 1000ml of milli-Q water and pH adjusted to 5.8± 0.02 using potassium hydroxide solution. A 0.45 µ filter using vacuum filtration was used to filter the resulting solution and the solution was then subjected to sonication for 15 min.

Preparation of Stock solution

The stock solution was made by weighing 10mg each of ofloxacin and ornidazole working standard accurately and separately into two 10ml volumetric flasks. They were then dissolved in methanol and the resulting solutions were sonicated and volume made upto the mark using methanol to acquire a solution of 1000 µg/ml of each drug. 1ml was withdrawn from each standard stock solution, shifted into separate 10ml volumetric flask and the final volume was made up to the mark using methanol(100 µg/ml- Stock solution A). 0.8ml was withdrawn from each stock solution A into separate 10ml volumetric flask and the final volume was made up to the mark using the mobile phase (8 µg/ml-ofloxacin and ornidazole.)

A reversed- phase high- performance liquid chromatographic system using isocratic elution mode with a mobile phase of acetonitrile: 10mM ammonium acetate buffer, with the pH adjusted to 5.8 with potassium hydroxide (25:75 v/v) with the addition of 1% trieethylamine to the aqueous phase and 10% methanol to the organic phase on C_{18} Phenomenex Monomeric 130A (250 × 4.6mm, 5µm) with 1ml/min flow rate at 295nm and 311nm with PDA and UV-Vis detector was used to carry out the HPLC analysis.

Calibration curves for OFL and ORN

Tablet formulation contains OFL: ORN in ratio of 2:5. Required aliquots of OFL and ORN stock solutions were transferred in 10ml volumetric flask and diluted up to the mark using the mobile phase for concentrations of 2-16µg/ml for both ofloxacin and ornidazole. The volume solutions injected were 20µl and chromatograms were aptly recorded. A computation of regression equations was done for both the drugs and average peak

Table 1: Linear Regression data for Calibration curves.

Parameters	Ofloxacin	Ornidazole
Range of Linearity (µg/ml)	2- 16	2-16
Correlation coefficient	0.9993	0.9991
Slope	95462	50655
y- intercept	32202	18861

areas versus concentrations were plotted to construct calibration curves (Table 1).

Marketed Formulation Analysis

Twenty tablets were weighed and their average weight was calculated. The same tablets were then powdered and transferred into a 100ml volumetric flask with a weight equivalent to 20 mg of ofloxacin. Then the powder was dissolved using methanol and the solution obtained was subjected to sonication and volume made upto the mark using methanol to gain a concentration of 200µg/ml of ofloxacin. The solution was filtered via a 0.45 µ filter using vacuum filtration. 1ml from the above solution was withdrawn and displaced into a 10ml volumetric flask; volume was made up using mobile phase. 1ml from the above solution was withdrawn and displaced to 10ml volumetric flask; volume was made up with the help of the mobile phase and injected in the HPLC system (20µg/ml OFL and 50µg/ml ORN). A volume of 20µl of the solution obtained above was injected into the HPLC system and the areas of the peaks obtained under the optimized chromatographic conditions were measured.

Method Validation

The analytical approach was validated for the parameters such as accuracy, linearity, precision, detection limit, quantization limit and robustness as per the recommendations of ICH Q2 (R1). The method's accuracy was estimated by measuring OFL and ORN's percentage recovery. The recovery studies were conducted by utilizing the same method for the drug sample to which documented amounts of OFL and ORN equivalent to 80, 100 and 120 percent of the label claim had been inserted (standard addition method). Three determinations were carried out at each level of the amount and the obtained results were then compared.

Intraday and inter day OFL and ORN precision analysis was conducted by calculating the related responses three times at different time intervals on the same day and on three separate days for a sample containing 8µg/ml of OFL and ORN. The limit of detection (LOD) and limit of quantitation (LOQ) were estimated by utilizing the given formulae: LOD= 3.3(SD)/S and LOQ= 10 (SD)/S, where SD=standard deviation of response (peak area) and S= average of the slope of the calibration curve.

System suitability testing is a vital part of the chromatographic method and is useful in verifying the chromatographic system reproducibility. To assess its efficacy, some system suitability test parameters have been tested by repeated injections of the solution containing drug sample at the concentration of 8µg/ml OFL and ORN to verify the system's reproducibility and the results are shown in Table 2.

A few parameters such as column oven temperature, wavelength, flow rate and pH of mobile phase were purposefully changed for the evaluation of robustness of the HPLC method. At one time, one factor was modified for estimating the effect. With respect to optimized parameters, each factor was assessed at three different levels (-2, 0, +2). Method robustness was conducted at the concentration level of 8µg/ml for both OFL and ORN.

Forced degradation studies

Forced degradation studies of bulk and tablet formulation were performed under acid and alkali hydrolysis, thermal, oxidative and photolytic degradation. Forced degradation in acid media was carried out by weighing 10mg of OFL and ORN in first 50ml round bottom flask with the addition of 50ml of 0.1M hydrochloric. These mixtures were administered to room temperature for 8 hrs. Similarly, forced degradation in alkali media was carried out by adding 50ml of 0.1M sodium hydroxide and kept for 8

Table 2: Summary of System suitability and Validation Parameters.

Parameters	Ofloxacin	Ornidazole
Retention time ± allowable time (m)	4.28± 0.03	6.75 ± 0.3
Theoretical Plates	4852.55	7829.49
Tailing factor (asymmetry factor)	1.55	1.08
Range of Linearity (µg/ml)	2-16	2-16
Correlation coefficient	0.9993	0.9991
LOD (µg/ml)	0.331	0.36
LOQ (µg/ml)	1.005	1.092
Recovery (%)	101.37	100.52
Precision (%RSD)		
Inter-day (*n*=6)	0.38	0.52
Intra-day (*n*=6)	0.18	0.33
Robustness	Robust	Robust

hrs at room temperature. This procedure was repeated but this time by refluxing all the above solutions except for ornidazole in 0.1M sodium hydroxide. Degradation using hydrogen peroxide was carried out by weighing 10mg of OFL and ORN in separate 10 ml volumetric flasks and volume was made up using methanol. From each, 1ml was displaced into a 10ml volumetric flask and volume was made up using 30%v/v hydrogen peroxide solution (100µg/ml) for bulk drugs. These mixtures were administered to room temperature for 4 hrs. For thermal and photolytic degradations, bulk drugs were placed in a petri dish in an oven for 24 hr at 80°C and in direct sunlight for 4 hr, respectively.

All the obtained degraded samples solutions were then diluted using the mobile phase to gain a final concentration of 10µg/ml OFL and ORN for HPLC analysis. Following that, 20µl solution from the solutions obtained above were inserted into the HPLC system and examined using the previously mentioned chromatographic conditions.

Dissolution studies

The dissolution studies of Ofloxacin and Ornidazole fixed dose combination tablet was performed. For the dissolution procedure the Dissolution media was 900mL 0.1 N HCL prepared by diluting 8.25 ml of 37.5% HCL to 1000ml with water. The Dissolution apparatus used was IP Apparatus 1 (Paddle Apparatus) with a temperature of 37±5°C at a stirring speed of 50 rpm and at time intervals of 0, 10, 20, 30, 45, 60 min. Dissolution testing was performed at the above conditions for 6 different tablets in the dissolution media by withdrawing 5ml of the solution each time and replacing with 5ml of dissolution media and the percentage of the dissolved drug was estimated and the cumulative percentage of drug dissolved with time was plotted.

RESULTS AND DISCUSSION

The mobile phase consists of acetonitrile: 10mM ammonium acetate buffer, whose pH is adjusted to 5.8 with potassium hydroxide (30:70 v/v) along with the addition of 1% TEA to the aqueous phase and 10% methanol to the organic phase, at 1ml/min flow rate was optimized and it resulted in well-resolved and sharp peaks with a tailing factor less than 2 for OFL and ORN. (Figure 2). The retention times of OFL and ORN were 4.278 min and 6.75 min respectively. Wavelengths of 295nm and 311nm were selected as the detection wavelengths for Ofloxacin and Ornidazole respectively. The calibration curve for OFL and ORN was seen to be linear when calculated over the range of 2- 16µg/ml. The calibration curves data is shown in Table 1. The provided method was effectively used on the tablet dosage form to estimate Ofloxacin and Ornidazole. The findings obtained from the combination were equivalent to the corresponding quantities labelled. The method developed was able to distinguish other excipients that are present in the tablet from the two drugs and was therefore found to be specific (Figure 3).

The Limit of Detection for OFL and ORN was estimated to be 0.331µg/ml and 0.36µg/ml, respectively, while Limit of Quantification was 1.005µg/ml and 1.092 µg/ml, respectively. The validation report and system suitability test parameters report data are presented in Table 2. The

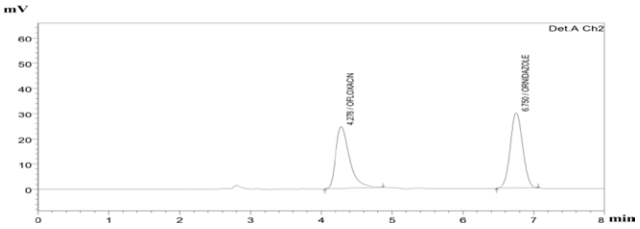

Figure 2: Optimised Chromatogram of OFL and ORN (API mixture).

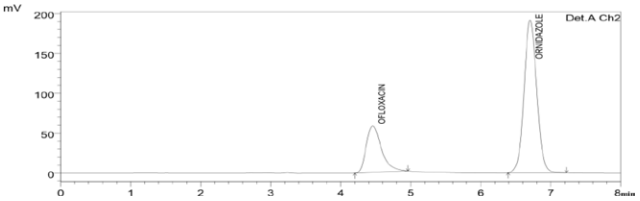

Figure 3: Chromatogram of tablet mixture.

Table 3: Stress testing report of Standard.

Sr. No.	Type of degradation	Stress Conditions	% degradation observed (%)	
			Ofloxacin	Ornidazole
1	Acid hydrolysis	0.1M HCl at 80°C for 4 hr	3.88	27.18
2	Alkali hydrolysis	Refluxed with 0.1M NaOH for 4 hr	4.38	22.21
3	Oxidative degradation	30% v/v hydrogen peroxide at room temperature for 4 hr	5.72	4.50
4	Photolytic degradation	Exposed to direct sunlight for 4 hr	88.84	89.42
5	Thermal degradation	Administered to 80°C in a hot air oven for 24 hr	1.53	1.51

summary report of Stress testing of Standard and Tablet are presented in Table 3.

Ofloxacin showed significant photolytic degradation with the generation of excess peaks as Figure 4 but was stable under alkali, acid and oxidative hydrolysis after refluxing at 80°C for 8 hr and 80°C in the hot air oven for 24 hr (Figures 5-8). Ornidazole was shown to be susceptible to alkali hydrolysis with its peak completely degrading and was also seen to show photolytic degradation as shown in Figures 5 and 7 respectively but was stable under acid and oxidative hydrolysis as well 80°C in the hot air oven for 24 hr (Figures 5,7,8).

The % drug delivery was seen to be higher than 80% in 20 min for all the evaluated products in case of dissolution studies. The Dissolution observation report for Ofloxacin and Ornidazole is given in Tables 4 and 5

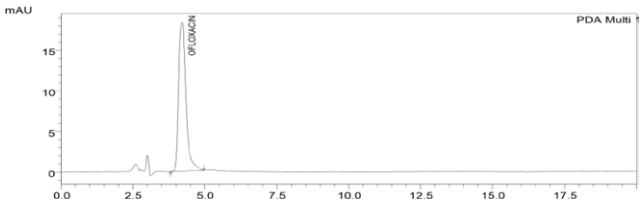

Figure 6: Chromatogram of standard exposed 0.1M sodium hydroxide after refluxing for 4 hr.

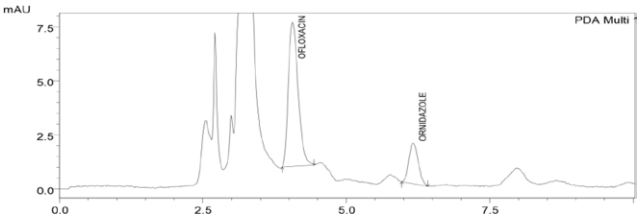

Figure 4: Chromatogram of standard exposed to light after 4hr.

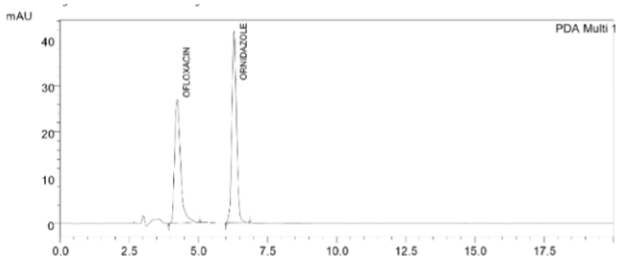

Figure 7: Chromatogram of standard in 30%v/v H_2O_2 solution after 4hr.

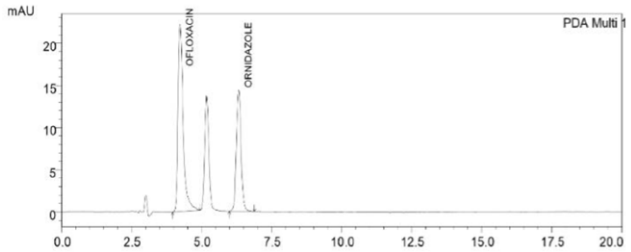

Figure 5: Chromatogram of standard exposed in 0.1M hydrochloric acid after refluxing for 4hr.

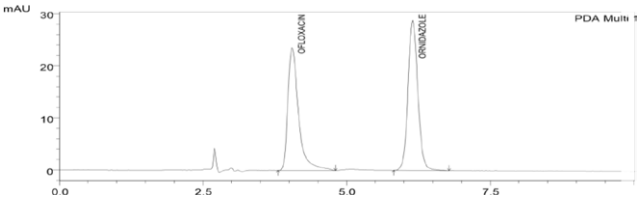

Figure 8: Chromatogram of standard exposed to heat after 24 hr.

Table 4: Ofloxacin dissolution observation report						
Sr. No	**Time points (m)**	**Area obtained (Average of 6 tablets)**	**S.D ±**	**%C.V**	**Sample Area/ Std Area**	**%Dissolved**
1	10	5754141.33	1306153.9	22.7	2.8	25.27
2	20	20670005.17	1228045.11	5.94	10.1	90.77
3	30	22627826	224391.47	0.99	11.01	99.37
4	45	22182921.67	1057278.62	4.77	10.79	97.41
5	60	23900745.17	1960935.85	8.2	11.63	104.96

Table 5: Ornidazole dissolution observation report.						
Sr. No	**Time points (m)**	**Area obtained (Average of 6 tablets)**	**S.D.**	**%C.V**	**Sample Area/ Std Area**	**%Dissolved**
1	10	7178163.17	1633624	22.76	2.83	25.63
2	20	25040062.3	1653505.33	6.6	9.88	89.39
3	30	27457169.5	286574.57	1.04	10.84	98.02
4	45	27633851.5	284328.97	1.03	10.91	98.65
5	60	27378323	2182355.34	7.97	10.81	97.74

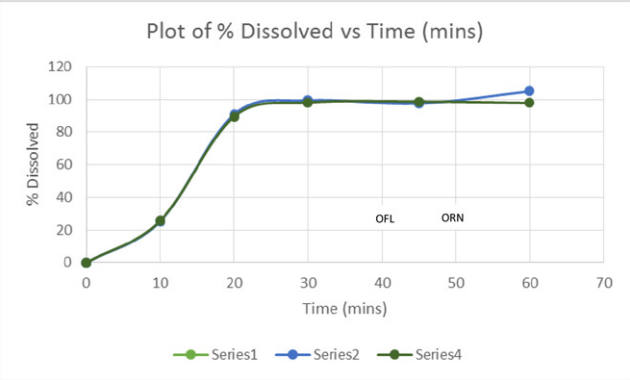

Figure 9: Plot of % Ofloxacin and Ornidazole dissolved vs time (mins).

respectively and the plot of % drug dissolved with time is shown in Figure 9.

CONCLUSION

In the proposed study, a precise, simple, accurate, sensitive and cost effective stability indicating assay method for simultaneous estimation of Ofloxacin and Ornidazole in bulk drug by RP-HPLC was established. The results obtained by analysing the forced degraded samples depicts that there was no other co-eluting peaks of interference due to variable stress components with the main peaks and the method was seen to be specific for the determination of Ofloxacin and Ornidazole amongst various degradants. The method was successfully used for assay and dissolution studies for tablet formulation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

mm: Millimeter; **nm:** Nanometer; **L:** Liter; **ml:** milliliter; **µl:** microliter; **G:** Gram; **Mg:** milligram; **µg:** milligram; **mol:** Molarity; **mM:** millimolar; **w/v:** weight/ volume; **w/w:** weight/ weight; **v/v:** volume/ volume; **min(s):** Minutes; λ**:** Wavelength; **µ:** Micron; **%:** Percentage; **ºC:** degree Celsius; **hr(s):** Hours; **SD:** Standard deviation; **RSD:** Relative Standard deviation standard deviation; **R2:** Correlation coefficient; **NMT:** Not more than; **NLT:** Not less than; **LOD:** Limit of detection; **LOQ:** Limit of quantification; **Rt:** Retention time; **OFL:** Ofloxacin; **ORN:** Ornidazole; **TEA:** Triethylamine; **AR:** Analytical grade; **ACN:** Acetonitrile; **$NH_4CH_3CO_2$:** Ammonium acetate; **NaOH:** Sodium hydroxide; **H_2O_2:** Hydrogen peroxide; **ICH:** International Council on Harmonization; **FDA:** Food and Drug Admintration; **RP:** Reverse Phase; **HPLC:** High Performance Liquid Chromatography; **UV:** Ultraviolet; **IR:** Infrared; **PDA:** Photo diode array; **API:** Active Pharmaceutical Ingredients; **IUPAC:** International Union of Pure and Applied Chemistry; **USP:** United States Pharmacopoeia; **IP:** Indian Pharmacopoeia; **BP:** British Pharmacopoeia; **C_{18}:** Octadecyl.

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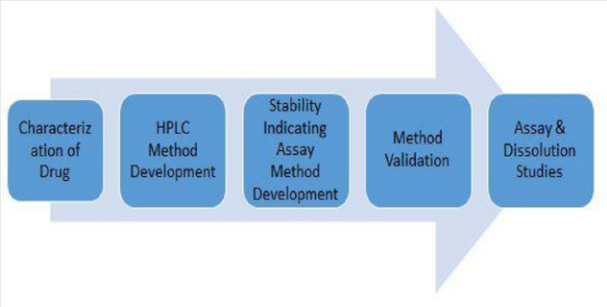
PICTORIAL ABSTRACT



SUMMARY

A precise, simple, accurate, sensitive and cost effective stability indicating assay method for simultaneous estimation of Ofloxacin and Ornidazole in bulk drug by RP-HPLC was established. The forced degraded sample results were then analysed and it was seen that there was no other co-eluting peaks of interference due to variable stress components with the main peaks and the method was seen to be specific for the determination of Ofloxacin and Ornidazole amongst various degradants. The method was then successfully used for assay and dissolution studies for tablet formulation.

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