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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF OFLOXACIN AND ORNIDAZOLE IN PRESENCE OF ORNIDAZOLE IMPURITY IN COMBINED PHARMACEUTICAL DOSAGE FORM

Farrel Lisa Gauncar* and Sachi S. Kudchadkar

Department of Quality Assurance, Goa College of Pharmacy, Panaji-Goa-403001, India.

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***Corresponding Author**
Farrel Lisa Gauncar
Department of Quality Assurance, Goa College of Pharmacy, Panaji-Goa-403001, India.

ABSTRACT

A simple, rapid, accurate and precise RP-HPLC method has been developed for simultaneous estimation of Ofloxacin and Ornidazole and Ornidazole Impurity A in combined dosage form. The column used was Phenomenex C18 (250 mm×4.6 mm, 5µm) with a flow rate of 1.0 ml/min using UV detection at 318 nm. The chromatographic separation was carried out using mobile phase containing ACN: Phosphate buffer at pH 3.0 adjusted with OPA in the ratio of 40:60 v/v. The described method was validated and linearity was observed over a concentration range of 2-20 µg/ml for Ofloxacin, 5-50 µg/ml for Ornidazole and 0.5-2 µg/ml for Impurity A with correlation coefficient of (R^2>0.999). All components were well resolved within 5 minutes. The proposed method can be applied effectively during routine purity testing and successfully for determination of Ofloxacin, Ornidazole and Ornidazole Impurity A in bulk and pharmaceutical dosage form.

KEYWORDS: Ofloxacin, Ornidazole, ORN Impurity A, RP-HPLC, Validated.

INTRODUCTION

Ofloxacin is a second generation fluoroquinolone being a broader-spectrum analog of norfloxacin, useful in treatment of various bacterial infections like pneumonia, UTIs etc. Chemically it is 7-fluoro-2-methyl-6(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1-azatricyclo (7.3.1.0) (5, 13) trideca-5(13), 6. 8, 11 tetraene-11 carboxylic acid. It exerts its bacterial effect on susceptible microorganisms by entering the bacterial cell and inhibits DNA gyrase, a type

2 topoisomerase and topoisomerase 4 an enzyme that seperates replicated DNA, thereby inhibiting bacterial cell division.[1]

Ornidazole belongs to nitroimidazole class of drugs namely tissue amoebicides. Chemically it is 1-chloro-3-(2-methyl-5-nitro-1H-imidazol-1-yl) propan-2-ol. It causes damage to the DNA strands or inhibits their synthesis. It is used in treatment of intestinal amoebiasis, hepatic amoebiasis.[2]

The combination is accepted and prescribed worldwide for broader spectrum of antibacterial activity. Ofloxacin and Ornidazole are official in USP and IP Literature review reveals that Ofloxacin is estimated by spectrophotometry, HPLC and spectroflourimetry. Both these drugs are available in combined tablet dosage form (200 mg and 500 mg) as an antiamoebic.[3]

The impurities in drug products can be attributed not only to the drug substance or inert ingredients used for formulating a drug product but they can also be brought into the drug product through the formulation process or by contact with packaging of the various impurities that can be found in drug products.[4,5]

“Any component of the drug product that is not the chemical entity defined as the drug substance or an excipient in the drug product.” (ICH Q6A: Specifications).

It is important to give greater consideration to these detrimental impurities. The presence of these impurities can cause toxicological problems. The presence of these unwanted chemicals, even in small amounts may influence the efficacy and safety of the pharmaceutical products.

The different pharmacopoeias such as BP, USP, and IP and so on are slowly incorporating limits to the allowable levels of impurities present in APIs or formulations.

An attempt was made to develop a simple, rapid, accurate and precise RP-HPLC method to Separate and quantitatively estimate OFX, ORN and ORN Impurity A in combined dosage form.

The drugs were selected for analytical method development based on the literature review which revealed that there was one Impurity method in combination with OFX and ORN and

other one with ORN as single component [6].The present work was carried out on Phenomenex-C18 column (4.6 mm×250 mm) with 5 µm particle size with flow rate of 1 ml/min using PDA detection at 318 nm. The method was validated as per ICH guidelines Q2 R1 using ACN and Phosphate buffer (20 mM) with pH 3.0 adjusted with OPA in the ratio of 40:60 v/v.

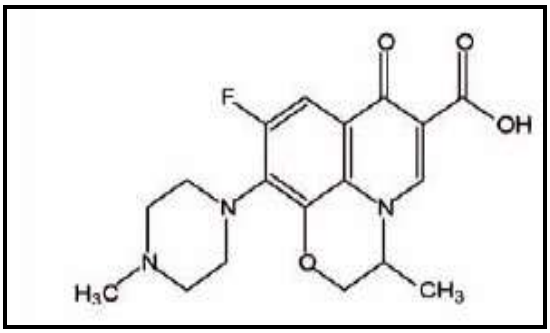

Fig 1: Chemical structure of OFX.

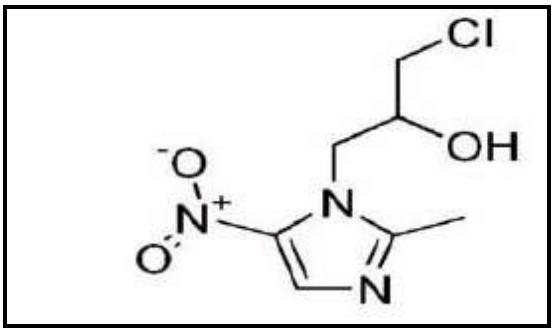

Fig 2: Chemical structure of ORN.

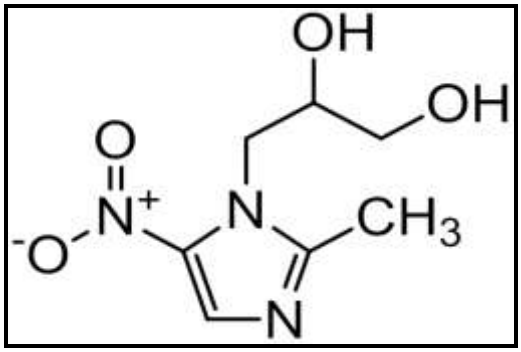

Fig 3: Chemical structure of ORN Impurity A.

Experimental

MATERIALS AND METHODS

Instrumentation and Chromatographic Conditions

The developed method was performed on Agilent HPLC 1200 series with PDA detector. Data

acquisition was carried out using Open Lab software. Chromatographic separation was performed using Phenomenex column C18 (250 mm×4.6 mm) 5 µm particle size at room temperature. The mobile phase used in this study was ACN and Phosphate buffer in the ratio of 40:60 v/v adjusted to pH 3.0 with OPA. All analyses were performed under isocratic condition at a flow rate of 1 ml/min. The mobile phase was filtered through 0.45 µm filtration unit (Millipore) before use and degassed using sonicator (Citizon). The pH of the mobile phase was adjusted with pH meter (Labtronics). All weighing was carried out using wensar electronic balance MAB 220.

Chemicals and Reagents

Ofloxacin (OFX) and Ornidazole (ORN) were provided by Vaishali Pharma Pvt. Ltd. Mumbai and Cadila Healthcare Pvt. Ltd. Kundaim-Goa respectively. ORN Impurity A was provided by Vergo Pharma Research, Verna-Goa as a gift sample. The marketed formulation was procured from local market.

Acetonitrile (ACN) and Methanol was procured from Rankem and Water HPLC grade was procured from water purification system (BIOAGE). Potassium diydrogen phosphate and Ortho- phosphoric acid were analytical reagent grade.

Diluent selection

Solubility studies were carried out for two drug entities and one impurity during its method development phase. Bulk drugs and impurity showed better solubility comparatively in ACN than other solvents and hence it was used as a solvent.

Preparation of OFX and ORN standard stock solutions

Accurately 100 mg of OFX and ORN were weighed and transferred in 100 ml of volumetric flasks and were dissolved separately in ACN to get each concentration of 1000 µg/ml. From this 10 ml was withdrawn and transferred to 100 ml of volumetric flasks and were diluted up to the mark with mobile phase containing ACN: Phosphate buffer at pH 3.0 in a ratio of 40:60 v/v to obtain working standard solutions of 100 µg/ml.

Impurity A standard stock solution

Accurately 10 mg of Impurity A was weighed and transferred in 10 ml of volumetric flask and dissolved in ACN to get a concentration of 1000 µg/ml. From this 100 µg/ml and 1 µg/ml working standard solutions were prepared with the mobile phase containing ACN: Phosphate

buffer at pH 3.0 in a ratio of 40:60 v/v and these were used to prepare further required concentrations.

METHOD VALIDATION

The optimized method was validated for determination of OFX and ORN in presence Of ORN Impurity. The proposed method was validated according to ICH guideline Q2 R1 for System Suitability, Specificity, Linearity, LOD and LOQ, Sample Analysis, Accuracy, Precision, Robustness and Sample Stability.[7]

System Suitability

System Suitability test is used to verify that the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. Standard mixture of OFX and ORN and ORN Impurity A at 20 µg, 50 µg and 0.5 µg concentration of each were prepared with their respective working standard solution. The sample was injected six times and chromatograms were recorded. From the peak area % RSD was calculated to ascertain reproducibility. The parameters determined were theoretical plates, asymmetry and % RSD of retention time for each peak. The data is presented in table 1

Specificity

The specificity of the method was assessed by observing the chromatograms of standard solutions of both the drugs and impurity individually and as a mixture. Sample solution was also used to demonstrate absence of interference from the excipients. The peak area and the retention time were compared to evaluate specificity when all were injected at test concentration of µg/ml. The data is presented in table 2

Linearity and range

The linearity and range for OFX, ORN and ORN Impurity A was observed simultaneously by addition from their working standard solution. A good linearity range was observed in the concentration range of 2-20 µg/ml for OFX and 5-50 µg/ml for ORN with R^2=1 for both the drugs. The range for Impurity A was confirmed from 0.5-4 µg/ml as it showed linear relationship with R^2=1.00. The calibration curves were plotted against peak areas v/s concentration. The slope, intercept, regression equation and correlation coefficient were obtained for OFX and ORN and Impurity A. The sample solution was injected as a triplicate of each concentration. Calibration curve are illustrated in fig 6, 7 and 8. The linearity results are given in 3.

LOD and LOQ

Limit of detection (LOD)

The detection limit is expressed as = $\frac{3.3\,\sigma}{S}$

The slope was estimated from the calibration curve of sample and standard deviation was based on standard deviation of responses in calibration curve.

Limit of Quantification (LOQ)

The detection limit is expressed as = $\frac{10\,\sigma}{S}$

The slope was estimated from the calibration curve of sample and standard deviation was based on standard deviation of responses in calibration curve. LOQ for Impurity A was determined through visual examination of sample by analyzing the samples with known concentration and the minimum level at which analyte can be quantified was determined with acceptable accuracy and precision studies. The data is presented in table 4.

Analysis of Marketed Formulation

Ten tablets were weighed to get the average weight of tablets. A sample of powdered tablets equivalent to 100 mg of ORN was transferred in 100 ml of volumetric flask. About 50 ml of ACN was added to the flask and was kept for sonication for 30 minutes and volume was made up to the mark with same solvent to give 1000 µg/ml standard stock solution.

The contents were then filtered through Whatmann filter paper (No.45). From this 10 ml was withdrawn and transferred to 100 ml volumetric flask and was diluted up to the mark with mobile phase containing ACN: Phosphate buffer in a ratio of 40:60 v/v at pH 3.0 to obtain a concentration of 100 µg/ml.

From this working standard solution, 5 ml was transferred in a series of six 10 ml volumetric flask. The same solvent was added up to the mark to give a solution containing 20 µg/ml and 50 µg/ml of OFX and ORN respectively. The data is presented in table 5.

Accuracy

The accuracy of the method was conducted at three different concentration levels (80 %, 100 %, and 120 %) by recovery studies. The recovery studies were carried out in triplicate preparations from standard solution for OFX and ORN and ORN Impurity A. The solutions were prepared in triplicate and injected into the chromatograph. The % recovery for standard

solution for OFX and ORN and ORN Impurity A were calculated. The data is tabulated for OFX and ORN and ORN Impurity A in table 6

Precision

Repeatability study

Repeatability study for OFX and ORN was conducted on marketed formulation by performing assay with ORN Impurity A spiked at its LOQ level. The test concentration was 20 µg/ml and 50 µg/ml for OFX and ORN respectively and ORN Impurity A was spiked at its LOQ level. The sample solutions were injected six times into the chromatograph and chromatogram was recorded. The concentration and % content in the marketed formulation was analyzed for OFX, ORN and for spiked Impurity A. Repeatability results are given in table 7.

Intermediate precision

Intermediate precision study for OFX and ORN was conducted on marketed formulation by performing assay with ORN Impurity A spiked at its LOQ level on two different days. The test concentration was 20 µg/ml and 50 µg/ml for OFX and ORN respectively and ORN Impurity A was spiked at its LOQ level. The sample solutions were injected six times into the chromatograph and chromatogram was recorded. The data is illustrated in table 8 for both the drugs and 9 for ORN Impurity A.

Robustness

The evaluation of robustness study was conducted to check reliability of the method by making deliberate changes in the method. Robustness of this method was performed by intentionally modifying Composition of mobile phase (varied by $\pm$ 2), flow rate (varied by ± 0.1 ml/min), pH of the mobile phase (varied by ± 0.2), Column thermostat temperature (varied by ± 2). The data is illustrated in table 10.

Sample Stability

Stability study was carried out on standard mixture containing 20 µg/ml of OFX, 50 µg/ml of ORN containing ORN Impurity A spiked at its LOQ level and was diluted up to the mark with mobile phase ACN: Phosphate buffer pH 3.0 in the ratio of 40:60 v/v. The sample solution was injected into the chromatograph at the interval of 1hr for 3hrs. The peak areas are illustrated in table 11.

RESULTS AND DISCUSSION

Selection of wavelength

Standard solutions of 10 µg/ml of OFX and ORN and ORN Impurity A of 2 µg/ml were prepared from their respective standard stock solution. The solutions were scanned in the UV range of 200-400 nm and overlain spectra of all three components were obtained.

OFX-299 nm ORN-318 nm Impurity A-298 nm Isobestic point-304 nm

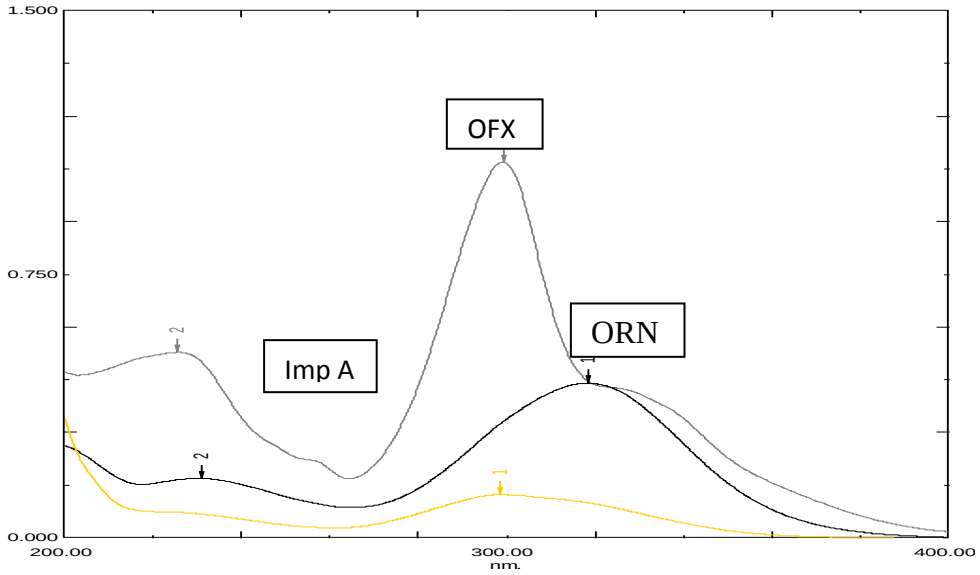

Fig. 4: Overlain spectra of OFX, ORN and ORN Impurity A.

The main criterion for selection of wavelength was based on absorption maxima of each component and isobestic point of OFX and ORN. Four wavelengths were selected for the study. The absorption maxima of Impurity A and OFX were observed at 298 nm and 299 respectively, but at these wavelengths the theoretical plates were less than 2000 and asymmetry was greater than 2 for OFX. The absorption maxima of ORN were observed at 318 nm and isobestic point was observed at 304 nm. At 304 nm both the drugs showed better response except ORN Impurity A and also asymmetry was greater at this wavelength as compared to 318 nm. At 318 nm all three components showed better response, good peak shape, better resolution, greater theoretical plates and asymmetry factor was within the limits. Hence 318 nm was selected for detection of OFX, ORN and ORN Impurity A in HPLC analysis.

Selection of mobile phase

The selection of mobile phase was carried out with various solvents like HPLC grade water, methanol and ACN on Phenomenex-C18 column (4.6 mm× 250 mm) with 5 µm particle

size. Standard solutions of OFX and ORN were prepared in a ratio of 2:5 and ORN Impurity A was prepared at 2 µg/ml concentration and diluted with the mobile phase.

Phosphate buffer solution (20 mM) was prepared at various pH like 2.7, 3.0 3.5 and 4.0.Trials were performed based on reported methods in the literature and then various ratios of mobile phase and pH were carried out. Further trials were carried out with other organic solvents such as methanol and ACN at various ratios, pH and flow rate. ACN: Phosphate buffer (80:20 v/v, 50:50 v/v, 40:60 v/v, 45:55 v/v), methanol: Phosphate buffer (55:45 v/v, 40:60 v/v, 45:55 v/v) were tried to select the mobile phase composition for further HPLC method development. It was observed that with methanol: Phosphate buffer (40:60 v/v) and ACN: Phosphate buffer (40:60 v/v) showed better results.

In case of methanol: Phosphate buffer it was observed that column showed high back pressures due to increase in aqueous phase. All three components showed good response with ACN: Phosphate buffer than with methanol: Phosphate buffer. The mobile phase was kept constant and trials were performed by varying the pH and flow rate. At lower pH 2.7 there was no proper resolution between the three components. As the pH was increased of the mobile phase there was significant tailing at pH 3.5 and 4.0. Hence pH 3.0 was selected as at this pH all three components were well resolved with good peak shape.

Further trials were carried out by altering the flow rate of the mobile phase wherein at 1.2 ml there was not much resolution between ORN Impurity A and ORN. Hence 1.0 ml flow per minute was selected in analysis of all three components at room temperature.

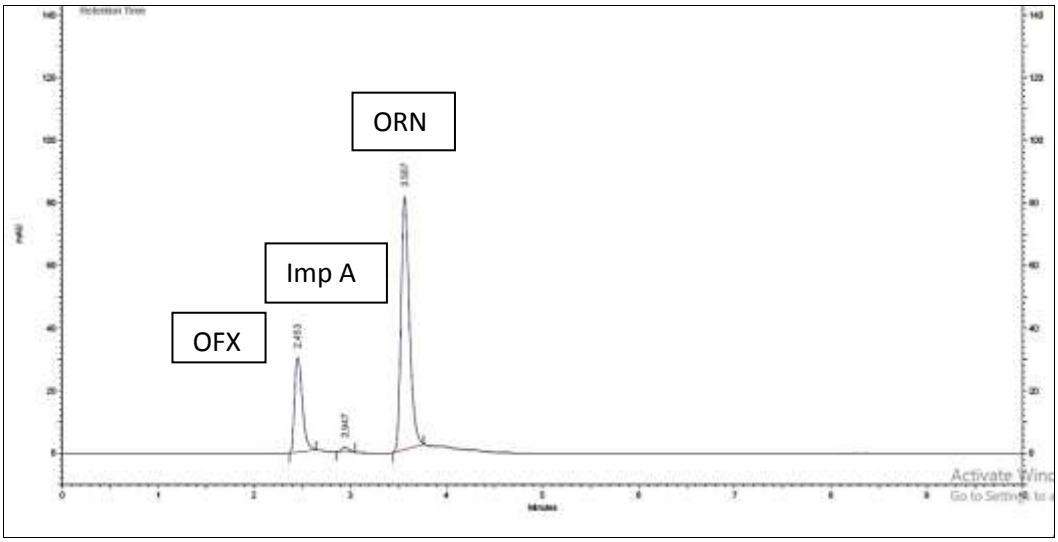

Fig 5: Chromatogram of OFX, ORN and ORN Impurity A in sample solution

System Suitability

System suitability parameters such as theoretical plates and asymmetry were within the acceptable limits and high resolution values were obtained which indicates complete separation of all three components. The results of system suitability are illustrated in table 1.

Table 1: Data of system suitability.

Sr. No	Parameters	Acceptance Criteria	OFX	Impurity A	ORN
1	% RSD of Rt	< 1%	0.45%	0.08	0.20
2	Average area	NA	24338989	558769	68704754
3	% RSD of area	< 2%	0.07	1.66	0.83
4	Theoretical plates	> 2000	4842	11460	8993
5	Asymmetry	< 2%	1.60	1.07	1.35
6	Resolution factor	> 1.5	-	3.705	4.77

Specificity

During specificity studies it was observed that there was no significant changes in their retention time and peak areas of OFX, ORN and ORN Impurity A from standard drug solution and sample solution. The peaks were well separated without any interference and hence the method was found to be specific. The results of specificity study are illustrated in table 2.

Table 2: Specificity results of standard and sample solution containing OFX, ORN and Impurity A.

Drugs and impurity	Standard solution		Sample solution	
	Peak Area (mAu*min)	Rt (min)	Peak Area (mAu*min)	Rt (min)
OFX	24273289	2.473	24151776	2.447
ORN	65277878	3.553	64244603	3.367
Impurity A	558565	2.947	568779	2.953

Linearity and Range

The method showed linearity across the concentration range of 2-20 µg/ml for OFX, 5-50 µg/ml for ORN and 0.5-4 µg/ml for ORN Impurity A with correlation coefficient 1 for all. Calibration curves were plotted against peak area v/s concentration for OFX, ORN and ORN Impurity A in fig 6, 7 and 8. Linearity results are given in table 3.

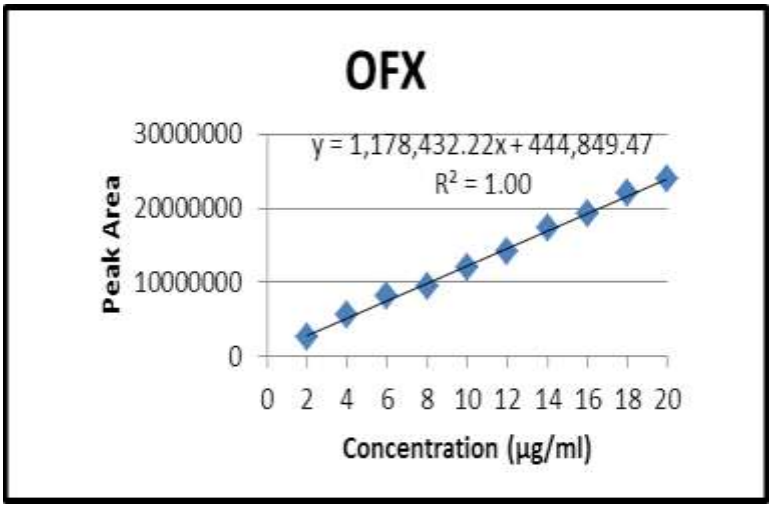

Fig. 6: Calibration curve of OFX.

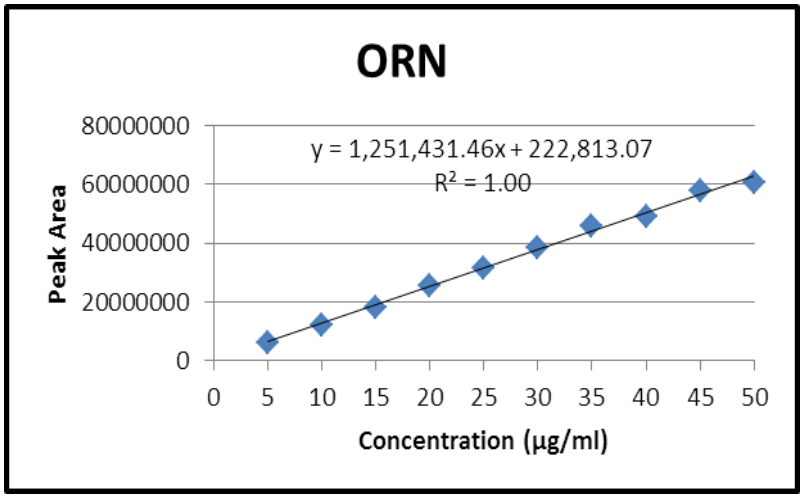

Fig. 7: Calibration curve of ORN.

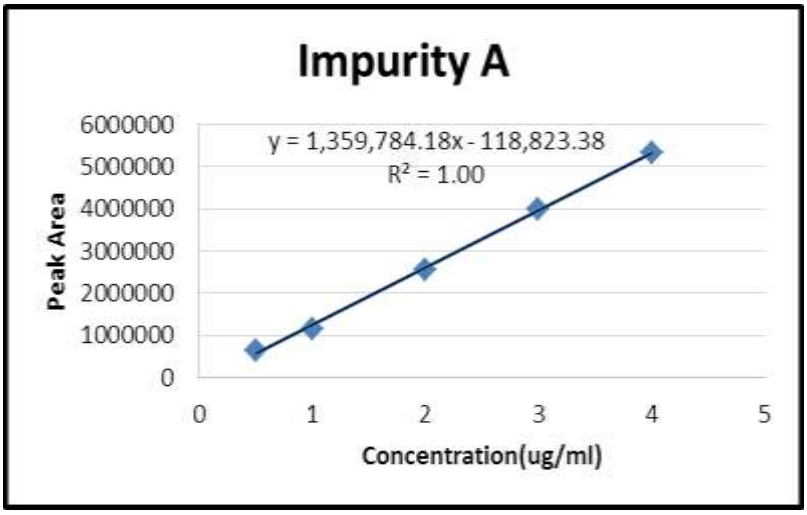

Fig. 8: Calibration curve of Impurity A.

Table 3: Results of linearity study.

Sr. No	Parameters	Results		
		OFX	ORN	Impurity A
1	Regression equation	y=1178432.22x+ 444849.47	y=1251431.46x+ 222813.07	y=1359784.18x- 118823.38
2	Slope	1178432.22	1251431.46	1359784.18
3	Intercept	444849.47	222813.07	118823.38
4	Correlation coefficient	R^2=1	R^2=1	R^2=1

LOD and LOQ

The limit of detection and limit of quantification for OFX, ORN and ORN Impurity A are illustrated in table 4.

Table 4: Data of LOQ and LOD values.

Results	OFX	ORN	Impurity A
LOD	1.14	3.41	0.26
LOQ	3.37	10.36	0.57

Analysis of marketed formulation

The % label claim for OFX and ORN in marketed formulation was found to be 101 % and 103 % for OFX and ORN respectively as per specified limits in the Indian Pharmacopoeia. The assay results on marketed formulation are illustrated in table 5.

Table 5: Analysis of marketed formulation.

Drug	Label claim	*Peak Area	Concentration of drug(µg/ml)	Content of drug(mg)	% label claim	% RSD
OFX	200 mg	24352696	20.287	202	101	0.07
ORN	500 mg	64843008	51.637	516	103	0.96

*Each value is a mean of six determinations.

Accuracy

The method was found to be accurate as all the % recovery results were between 98-104 %. The results obtained during recovery study for OFX, ORN and ORN Impurity A are listed below in table 6.

Table 6: Recovery data for OFX, ORN and ORN Impurity A.

Sample	Conc. of sample Solution (µg/ml)	Level of addition (Standard %)	Conc. of standard added (µg/ml)	Peak areas (mAu* Min)	Amount recovered (µg/ml)	% recovery
OFX	10	80	8	22575080	8.354	104.42
		100	10	24936537	10.005	100.05
		120	12	27748824	11.974	99.33
ORN	25	80	20	59436908	20.432	102.16
		100	25	65711827	25.112	100.44
		120	30	72109029	29.87	99.56
Impurity A	0.5	80	0.4	764286	0.394	98.66
		100	0.5	938733	0.503	100.6
		120	0.6	1111873	0.610	101.66

*Each value is a mean of three determinations.

Precision

The % RSD of sample solution containing OFX and ORN and concentration for Impurity A were found to be NMT 2% as per the acceptance criteria. The results for repeatability study are given in table 7 and Intermediate precision study are illustrated in table 8 for both the drugs and table 9 for ORN Impurity A.

Table 7: Repeatability data of OFX, ORN and Impurity A.

Sr. No	OFX		ORN		Impurity A	
	Area (mAu* min)	Conc. from equation	Area (mAu* min)	Conc. from equation	Area (mAu* min)	Conc. from equation
Mean	24222475	20.176	63879362	50.878	569831	0.505
SD		0.133		0.578		0.003
% RSD		0.66		1.14		0.60
% Assay		100.88		101.75		101.00

*Each value is a mean of six determinations.

Table 8: Intermediate precision data of OFX and ORN.

Sr. No	OFX				ORN			
	Area		Conc.(µg/ml)		Area		Conc.(µg/ml)	
	Day1	Day2	Day1	Day2	Day1	Day2	Day1	Day2
Mean	24222475	24230007	20.176	20.183	63879362	63843140	50.878	50.837
SD			0.133	0.1323			0.578	0.5382
%RSD			0.66	0.66			1.14	1.06
% Assay			100.88	100.91			101.75	101.67

*Each value is a mean of six determinations.

Table 9: Intermediate precision data of Impurity A.

Sr. no.	Impurity A			
	Area		Conc.(µg/ml)	
	Day 1	Day 2	Day 1	Day 2
Mean	569831	569569	0.505	0.506
SD			0.003	0.0030
% RSD[41]			0.60	0.61
% Assay			101	101.2

*Each value is a mean of six determinations.

Robustness

The % RSD for OFX, ORN and ORN Impurity A was NMT 2 % as per acceptance criteria. Hence the method was found to be robust. The robustness study results are illustrated in table 10.

Table 10: Data of Robustness study for OFX, ORN and Impurity A.

Variation		Retention time (Rt)		
		OFX	ORN	Impurity A
Composition of mobile phase	38:62	2.513	3.580	3.017
	40:60	2.453	3.353	2.940
	42:58	2.460	3.450	2.907
pH of mobile phase	2.8	2.447	3.533	2.927
	3.0	2.453	3.353	2.940
	3.2	2.467	3.553	2.940
Flow rate	0.9	2.498	3.430	3.012
	1.0	2.453	3.353	2.940
	1.1	2.399	3.398	2.892
Column thermostat temperature	28 C	2.440	3.353	2.947
	30 C	2.453	3.353	2.940
	32 C	2.433	3.460	2.953

Sample Stability

The sample solution containing OFX, ORN and ORN Impurity A were found to be stable up to 3 hrs. The sample stability results are illustrated in table 11.

Table 11: Data of sample study of OFX, ORN and Impurity A.

Sr. no	Time (in hrs)	Retention time (mins)			Peak area (mAu*min)		
		OFX	ORN	Impurity A	OFX	ORN	Impurity A
1	0	2.460	3.547	2.933	24373647	65277872	557873
2	1	2.453	3.547	2.933	24273289	65271763	552682
3	2	2.487	3.593	2.967	24276832	65272643	551895
4	3	2.453	3.547	2.933	24151776	65168567	552468

SUMMARY AND CONCLUSION

A Simple, rapid, accurate and precise RP-HPLC method was developed for estimation of OFX and ORN in presence ORN Impurity A in combined tablet dosage form. The solvent that was used in this study was ACN over methanol as high back pressures were observed with methanol on Phenomenex column C18 (4.6 mm×250 mm, 5 µm) and also all three components showed better response with ACN than methanol. However mobile phase containing ACN: Phosphate buffer at pH 3.0 in a ratio of 40:60 v/v ratio was selected for detection and quantification of all three components at 318 nm. All three components were eluted within 5 minutes run time with good peak shape, resolution and greater theoretical plates. The developed HPLC method can be used for purity testing of OFX and ORN in presence of ORN impurity A in bulk and pharmaceutical dosage form

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