Development and Validation of an RP-HPLC Method for Simultaneous Analysis of Ofloxacin and Ornidazole in Tablets

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ABSTRACT

A simple, sensitive, and inexpensive high performance liquid chromatographic method has been developed for simultaneous determination of ofloxacin and ornidazole in pharmaceutical formulations. Chromatographic separation was achieved on a BDS-C_{18}-Hypersil column (250 mm x 4.6 mm i.d., 10 µm). Mobile phase was 80% water (containing 0.55 ml/L of triethylamine as peak modifier) and 20% acetonitrile; final pH was adjusted to 3.0 with orthophosphoric acid. Detection was done at 284 nm. Response was a linear function of concentration in the range 1–20 µg/ml for ofloxacin and 2.5-50 µg/ml for ornidazole; the correlation coefficients were 0.9998 and 0.9995, respectively. The limit of detection were 0.01 and 0.02 µg/ml for ofloxacin and ornidazole respectively, where as limit of quantitation were 0.05 and 0.1 µg/ml . The accuracy result for ofloxacin and ornidazole at eighty percent drug (80%), hundred percent (100%), and one hundred and twenty percent (120%) were ranged from 99.6-100.9%. The inter- and intra-day precision was less than 1%. Total elution time for the two components was less than 9 min.

Keywords: RP-HPLC, Ofloxacin, Ornidazole, Validation.

INTRODUCTION

Ofloxacin (OF, Fig. 1a) ((*RS*)-9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro- 7H-pyrido(1,2,3-de)-1,4-benzoxazine-6-carboxylic acid) is a totally synthetic fluoroquinolone antimicrobial agent with a broad spectrum of activity against both gram-positive and gram-negative bacteria, as well as atypical pathogens such as *Mycoplasma*, *Chlamydia* and *Legionella* [(1)]. The mechanism of the bactericidal effect of OF is based on the inhibition of the DNA gyrase of the bacteria, the enzyme that produces a negative supercoil in DNA and thus permits transcription and replication [(2)]. Drug dosage forms containing OF are used for the treatment of gastrointestinal, pulmonary, urinary, sexually transmitted diseases, as well as for prevention of infection in the immune-compromised patient [(3)]. Ofloxacin is official in BP [(4)], USP [(5)] and EP [(6)]. The assay procedure mentioned in these pharmacopoeias uses non-aqueous titration. Some methods such as microbiological assay [(7)], capillary electrophoresis [(8)], flow-injection chemiluminescence [(9, 10)], spectrofluorimetry [(11)], phosphorimetry [(12)], were developed for determination of OF in pharmaceutical formulations.

Ornidazole (OZ, Fig. 1b), a 5-nitro-imidazole derivative has anti-amoebic and antimicrobial activities [(13, 14)], chemically; OZ is 1-chloro-3-(2-methyl-5-nitroimidazole-1yl)-propan-2-ol. Tablet dosage form containing ofloxacin (200mg) and ornidazole (500 mg) are available in market (i.e. Ornida® and Ornof®). HPLC method for the estimation of ornidazole has been reported in pharmaceutical preparations and body fluids [(15, 16, 17)].

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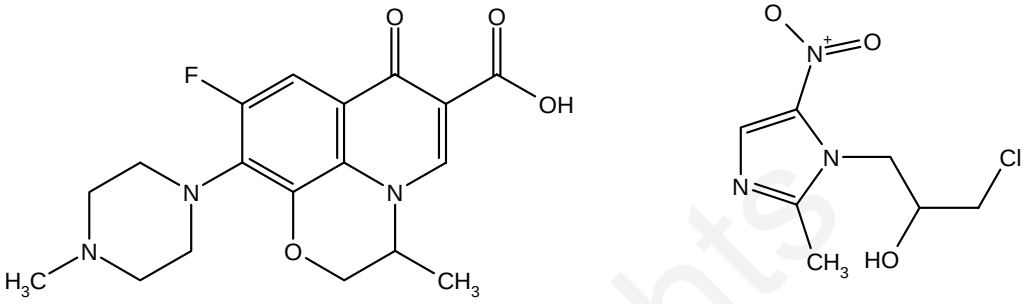
HPLC methods [18, 19] for the estimation of ofloxacin and ornidazole in tablets have been reported. The linearity range for ofloxacin and ornidazole were 40-140 µg/ml, 100-350 µg/ml, respectively. A validated spectrophotometric method for simultaneous estimation of ofloxacin and ornidazole in tablet dosage forms also has been reported [20]. A validated HPTLC [21] method also been reported for the estimation of ofloxacin and ornidazole in tablet dosage forms. The calibration curve range was 1-5 and 2.5-12.5 µg/ml for ofloxacin and ornidazole.

Form the literature survey it was noticed that the reported methods are less sensitive and having narrow range of linearity [18-20]. The objective of the present method was to develop sensitive, specific, precise and accurate method for the estimation of ofloxacin and ornidazole in tablet formulation. The present paper describes validated HPLC method for the simultaneous estimation of OF and OZ in tablet formulation.



a) b)

Figure 1 : a) Ofloxacin b) Ornidazole

EXPERIMENTAL

Material and Methods

Reference standards of ofloxacin and ornidazole were obtained from Glenmark Pharmaceuticals, Mumbai (India). A pharmaceutical product (Ornida®, Batch No. 1750019, containing active pharmaceutical ingredients- ofloxacin 0.20 g and ornidazole 0.50 g) were purchased from Local Indian Pharmacy Shops. Acetonitrile, water (HPLC grade), ortho-phosphoric acid, and triethylamine (analytical reagent grade) were purchased from Merck (Darmstadt, Germany).

Apparatus and Chromatographic Conditions

For chromatographic analysis, Merck-Hitachi (Lachrome®) HPLC equipped with quaternary gradient pump (Lachrome® 7100), UV detector (Lachrome® 7400), an auto-sampler (L7200) with a rheodyne injector (Rheodyne 7125) holding 100µl loop was used. The signals were acquired and analyzed using Windows 2000 based, D700 HSM chromatography data station software. The separation of the compounds was made on a BDS-Hypersil-C-18 Column (250mm×4.6 mm, 10 µm, Thermo Electron Corporation, USA) at ambient temperature. The mobile phase used in this study was 80% water (containing 0.55ml/L triethylamine) and 20% acetonitrile (v/v); final pH adjusted to 3.0 with orthophosphoric acid. All analyses were performed under isocratic condition at a flow rate of 1.0 ml/min. The UV detection was carried out at 284 nm for acquiring the signals. Mobile phase was filtered through 0.45 µm filteration unit (Millipore Corporate, USA) before use and degassed in an ultrasonic bath. The pH meter used was Orion Research (model 611, Orion Research incorporation, USA).

Standard Preparation

About 50 mg OF and 50 mg Oz were accurately weighed and taken in 100 ml volumetric flasks separately and dissolved in the mobile phase. Solutions were sonicated for 5 mins. The volume was adjusted to the mark with mobile phase to give stock solution of 500 µg/ml of OF and OZ separately. Calibration standards were prepared using the stock solutions.

Aliquots of standard stock solution of OF and OZ stock solution were taken in 10 ml volumetric flask and diluted to the mark with mobile phase to obtain final concentration of OF (1-20 µg/ml) and OZ (2.5-50 µg/ml). The linearity of the method was checked by analyzing these solutions in the range of 1–20 µg/ml for ofloxacin (1, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 µg/ml) and 2.5–50 µg/ml for ornidazole (2.5, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 µg/ml) and. Triplicate injections of 10 µl were made for each concentration of each drug separately and chromatographed.

To assess the system suitability of the system, standard solution of ofloxacin (10 µg/ml) and ornidazole (25 µg/ml) was prepared and analyzed. System suitability parameters (retention time, area, peak height, width, asymmetry, symmetry, efficiency and resolution) were evaluated.

Sample preparation

Twenty tablets were weighed and finely powdered in a pestle and mortar. Tablets powder equivalent to 10 mg of OF and 25 mg of OZ was transferred to 100 ml volumetric flask and dissolved in about 50 ml of mobile phase. The solutions were sonicated for 15 min., diluted to the mark with mobile phase and then filtered through 0.45 µm membrane filters (Millipore, USA). The concentration of the ofloxacin and ornidazole were 100 µg/ml and 250 µg/ml respectively. Aliquotes (4, 5 and 6 ml) of the sample solution were transferred to 50 ml volumetric flasks and diluted with mobile phase to obtain 8, 10 and 12 µg/ml of OF and 20, 25 and 30 µg/ml of OZ.

Method accuracy was determined by addition of known amounts of ofloxacin and ornidazole to a sample solution of known concentration and comparing calculated and measured concentrations. A sample solution containing ofloxacin (100 µg/ml) and ornidazole (250 µg/ml) was prepared by dissolving tablet triturate equivalent to 10 mg ofloxacin and 25 mg ornidazole in 100 ml mobile phase. Samples (5 ml) of the filtered solution were transferred to 50-ml volumetric flasks containing 4.0, 5.0 and 6.0 ml ofloxacin and ornidazole standard solution (100.0 and 250.0 µg/ml, respectively). The solutions were diluted to volume with mobile phase so the final concentrations were 8.0, 10.0 and 12.0 µg/ml for ofloxacin and 20.0, 25.0 and 30.0 µg/ml for ornidazole.

Alternatively to determine accuracy of the method and recovery of ornidazole and ofloxacin in tablet dosage form, samples of ofloxacin (200mg) and ornidazole (500mg) were prepared in triplicate at the 80%, 100%, and 120% levels of the target ofloxacin and ornidazole concentration and assayed according to the procedures.

To evaluate specificity, a synthetic mixture containing 100 mg ofloxacin, 250 mg ornidazole, and 30 mg each of starch, lactose, magnesium stearate and avicel, which are present as excipients in the pharmaceutical formulation, was accurately weighed and transferred to a 100 ml volumetric flask. The mixture was shaken well with about 50 ml mobile phase, sonicated and then diluted to volume with mobile phase. After filtration, 1 ml of the filtrate was transferred to a 100 ml volumetric flask and diluted to volume with mobile phase, to furnish a final solution containing 10.0 µg/ml ofloxacin and 25.0 µg/ml ornidazole. A placebo solution was also prepared omitting active pharmaceutical ingredients.

Construction of Calibration Plots

Solutions of both drugs having different concentrations were prepared by dilution of the stock solution. These solutions (10 µl) were chromatographed and the peak areas were measured. Peak areas were then plotted against the respective concentrations for both OF and OZ. From the plots it was found that the response was linear in the studies range (1.0-20.0 µg/ml) for OF and for OZ (2.5-50.0 µg/ml). Unknown assay samples were quantified by reference to these calibration plots.

Assay of Tablet Formulation

Twenty tablets were weighed accurately and the average weight was calculated. These tablets were triturated in pestle mortar to a fine power. The amount

equivalent to average weight of tablet was transferred to 500 ml volumetric flask and dissolved in the mobile phase. The solution was filtered through 0.45 µm Millipore disc filters. Replicate solutions of the required dilution were prepared from the stock solution. These solutions were filtered through 0.45 µm filter and then 10µl of samples were injected for quantitative analysis**.** The amounts of OF and OZ per tablet were calculated from the calibration plot.

Method Validation

The method was validated for linearity, accuracy, precision, repeatability and specificity. Accuracy was assessed by measuring recovery at three different levels, 80, 100 and 120% of the amount expected from analysis of the formulation, in accordance with ICH guidelines [22, 23]. Precision was assessed by measurement of intra and inter-day precision. In the intra-day study the concentrations of both drugs were calculated three times on the same day at intervals of 1 h. In the inter-day study the concentrations of the drugs were calculated on three different days. Specificity of the methods was judged by calculating recovery of each analyte in presence of pharmaceutical excipients. The limit of detection (LOD) is defined as the lowest concentration of an analyte that can be readily detected but not necessarily quantified. It is usually regarded as the amount for which the signal to noise ratio (SNR) is 3:1. The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte that can be quantified with acceptable precision and accuracy. It is usually regarded as the amount for which the SNR is 10:1. Two types of solution, i.e. blank and spiked with known progressively decreasing concentrations of each analyte, were prepared and analyzed. The limits of detection (LOD) and quantification (LOQ) were then established by evaluating the minimum levels at which the analyte could be readily detected or accurately quantified, respectively.

RESULTS AND DISCUSSION

Method Optimization

Conditions were optimized for simple, isocratic, accurate, and sensitive simultaneous HPLC determination of ofloxacin and ornidazole in tablet formulations. A large number of HPLC methods have been developed for analysis of ofloxacin but very few for ornidazole. Method development was started with 10% acetonitrile in water, but broad peaks were observed. The mobile phase 20:80 (acetonitrile: water) pH adjusted to 3.0 with orthophosphoric was prepared. This resulted in distorted signals that were not well defined. Addition of 0.55ml triethylamine in one liter of water (as peak modifier) and subsequent adjustment of pH of mobile phase with phosphoric acid resulted in good separation and symmetrical peaks. Addition of triethylamine improves the separation by masking polar silanol groups on the stationary phase, thus enabling analyte molecules to move through the column without interference from the stationary phase.

The optimum mobile phase was, therefore, 80% water (containing 0.55ml/L of triethylamine), and 20 % acetonitrile; pH adjusted to 3.0 with orthophosphoric acid. Under these experimental conditions sharp peaks were obtained for ofloxacin and ornidazole at the retention time 6.5 and 8.2 min, respectively, as shown in Fig. 2.

System Suitability

System suitability test is used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. In the present method, the percentage of coefficient of variation (%CV) of the retention time were 0.80, 0.66 and peak area 0.23 and 0.29, respectively. The percentage of coefficient of variation for other parameters was less than 1% (Data on file)

Linearity

The method was evaluated by determination of the correlation coefficient and intercept values. The results are given in Table-1. The representative calibration equation for ofloxacin and ornidazole were: *y=39069(±71.95) x -1830.6 (± 362.65); y =11042(± 11.20) x +172.92 (± 675.01)* respectively. The correlation coefficient was greater than 0.9995. From the calibration plots it was clear that the response was linear in the studied range (1 - 20 µg/ml for OF and 2.5-50 µg/ml for OZ) (Fig 3 and 4).

Table 1. Results from the study of linearity

Parameters	Ofloxacin	Ornidazole
Linearity range (µg/ml)	1-20	2.5-50.0
Slope ± SD	39069 ± 71.95	11042 ± 11.20
Intercept ± SD	-1830.6 ± 362.65	172.92 ± 675.01
Regression Coefficient (r2)	0.9998	0.9995
Tailing Factor	1.08	1.05
Resolution factor	2.60	
Limit of detection (µg/ml)	0.01	0.02
Limit of quantitation (µg/ml)	0.05	0.1

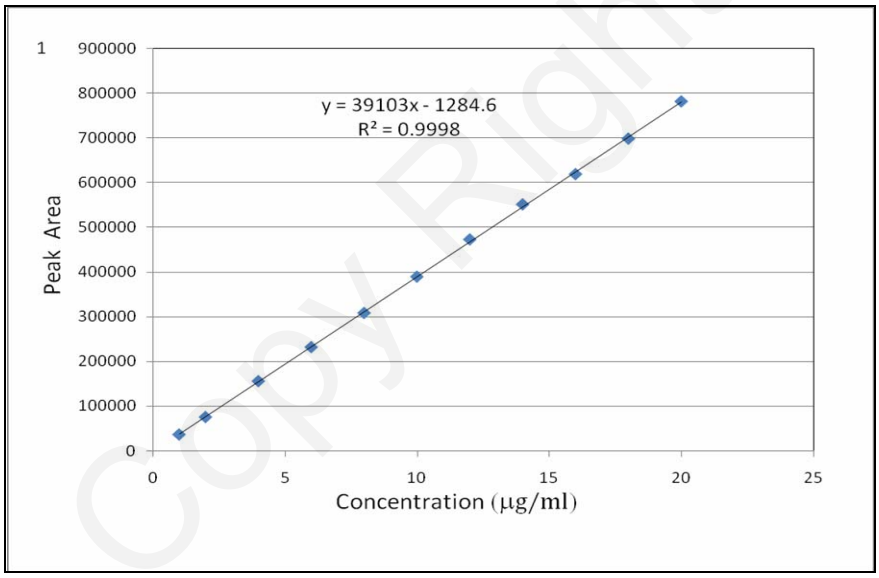


Fig. 3 : Calibration curve (of ofloxacin) shows linearity over the concentration range 1.0-20.0 µg/ml

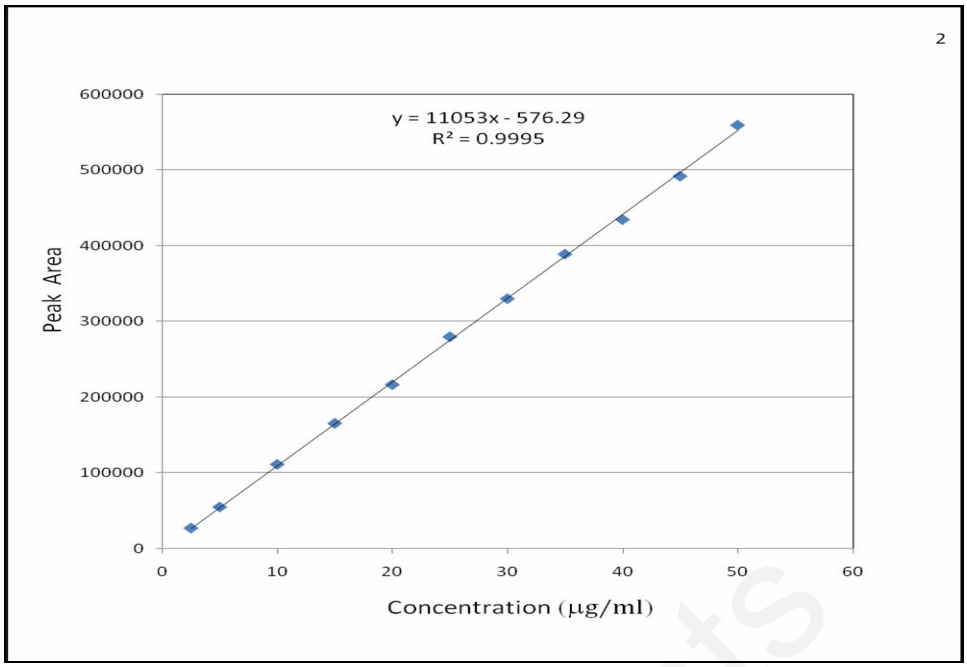


Fig. 4 : Calibration curve (of Ornidazole) shows linearity over the concentration range 2.5-50.0 µg/ml

Accuracy

Method accuracy was determined by addition of known amounts of ofloxacin and ornidazole to a sample solution of known concentration and comparing calculated and measured concentrations (n=6) as well as conducting a recovery study at three different concentrations (80, 100, and 120% of the amount expected from analysis of the formulation) by replicate analysis, in accordance with ICH guidelines (Table 2A, 2B). It is clear from the recovery study that the method is very accurate as the results were within the acceptable range, i.e. % R.E.< 2.0% and S.D. < 1.0%

Table 2 A : Accuracy of the method (based on standard addition method)

Drug	Amount of sample taken (µg/ml)	Amount added		Recovery	R.E. (%)
		%	(µg/ml)	Mean ± sd	
Ofloxacin	10.0	80	8.0	100.12 ± 0.38	0.12
		100	10.0	100.54 ± 0.68	0.54
		120	12.0	100.08 ± 0.58	0.08
Ornidazole	25.0	80	20.0	100.59 ± 0.26	0.59
		100	25.0	100.54 ± 0.73	0.54
		120	30.0	100.48 ± 0.55	0.48

Table 2 B: Accuracy of the method (Recovery method)

		Level 80%			**Level 100%**			**Level 120%**		
Drug	Wt of sample (mg)*	**775.1**	**785.22**	**773.25**	**980.23**	**960.76**	**971.25**	**1181**	**1175.6**	**1178.5**
Ofloxacin	Theo. Conc. (mg)	158.99	161.07	158.62	201.07	197.08	199.23	242.25	241.15	241.75
	measured conc. (mg)	159.11	160.29	157.29	200.65	199.26	202.12	240.15	240.21	244.12
	% recovery	100.07	99.52	99.16	99.79	101.11	101.45	99.13	99.61	100.98
	Mean	**99.58**			**100.78**			**99.91**		
	S.D.	0.46			0.88			0.96		
	%RE	**-0.42**			**0.78**			**-0.09**		
Ornidazole	Theo. Conc. (mg)	397.49	402.68	396.54	502.68	492.70	498.08	605.63	602.88	604.37
	measured conc. (mg)	399.15	399.98	400.10	503.69	499.18	503.12	606.12	609.73	612.73
	% recovery	100.42	99.33	100.90	100.20	101.32	101.01	100.08	101.14	101.38
	Mean	**100.22**			**100.84**			**100.87**		
	S.D.	0.80			0.58			0.69		
	%RE	**0.22**			**0.84**			**0.87**		

* Weight of tablet triturate, processed and analyzed as per method

Table 3: Intra-day and inter-day precision

Nominal Concentration (µg/ml)	**Intra-day (n=6)**			**Inter-day (n=3)**		
	Mean ± S.D.	**Precision (% CV)**	**R.E. (%)**	**Mean ± S.D.**	**Precision (% CV)**	**R.E. (%)**
Ofloxacin						
8	8.02 ± 0.018	0.219	0.179	7.97 ± 0.040	0.50	-0.375
10	10.08 ± 0.058	0.58	0.843	9.95 ± 0.041	0.41	-0.486
12	12.07 ± 0.075	0.624	0.595	11.90 ± 0.071	0.593	-0.798
Ornidazole						
20	19.90 ± 0.111	0.555	-0.507	19.83 ± 0.152	0.769	-0.857
25	25.08 ± 0.080	0.318	0.327	24.88 ± 0.072	0.288	-0.463
30	29.88 ± 0.177	0.592	-0.405	29.79 ± 0.143	0.48	-0.738

Precision

From the stock solution, mixed standard containing OF and OZ in the ratio of 2:5 was prepared. Six replicate sample solutions were prepared and injected. For the study of intra-day precision samples of both drugs were calculated 3 times on the same day at interval of 1 hr. In the inter-day precision study the samples was calculated on three consecutive days. From the peak area of OF and OZ the amount of each drug present in the sample was determined. Accuracy was expressed as relative error and precision was expressed as % CV. The intra-day and inter-day precision were determined and result of which are given in Table-3.. Intra-day precision was ranged from 0.219 to 0.624 and the relative error (bias, %) was between 0.179 and –0.507. Inter-day precision was between 0.288 to 0.769 and the relative error (bias, %) was –0.857 to –0.375. The intra- and inter-day precision was within range i.e. less than 2%, indicating the method enable precise quantitative analysis of both drugs.

Specificity

Figure 2. showed the complete separation of analyte peaks from the tablet excipients. The tailing factor was less than 2% and resolution factor was 2.6. The mean retention time for OF and OZ were found to be 6.58 ± 0.053 min., 8.26 ± 0.055 min respectively for six replicates. The peaks obtained for the drugs were sharp, symmetrical and have clear base line separation (Fig. 2A, 2E). The specificity of the method was tested by calculating the percentage recovery of each component in the presence of the other component and in the presence of possible interfering materials such as starch, lactose, magnesium stearate, and avicel. The results are presented in Table-4, which shows separation of analytes from the excipients was complete. The retention times of both drugs from standard solution (Fig 2 B and 2C) and from tablet solution were identical and no co-eluting peaks from the diluents were observed (Fig 2D). The resolution factor was 2.62. This indicates the method is specific for simultaneous determination of both drugs.

Table 4. Specificity of the method

Ofloxacin			**Ornidazole**		
Nominal concentration (µg/ml)	**Recovered (µg/ml)**	**Recovery (%)**	**Nominal concentration (µg/ml)**	**Recovered (µg/ml)**	**Recovery (%)**
10.0	10.19	101.90	25.0	25.24	100.96
10.0	10.15	101.50	25.0	25.12	100.48
10.0	10.18	101.80	25.0	25.20	100.80
Mean		**101.73**	**Mean**		**100.75**
S.D.		**0.208**	**S.D.**		**0.24**
%CV		**0.205**	**%CV**		**0.242**

Table 5. Result from assay of tablet formulation (Ornida®, 1750019**)**

Drug	**Label Claim (mg per tablet, n=6)**	**Amount Found (mg)**	**Drug Content (%)**	**S.D.**	**%CV**
Ofloxacin	200	202.18	101.09	0.398	0.394
Ornidazole	500	502.59	100.51	0.643	0.639

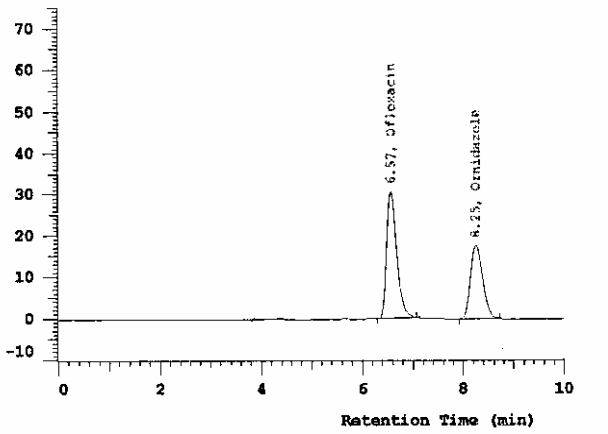
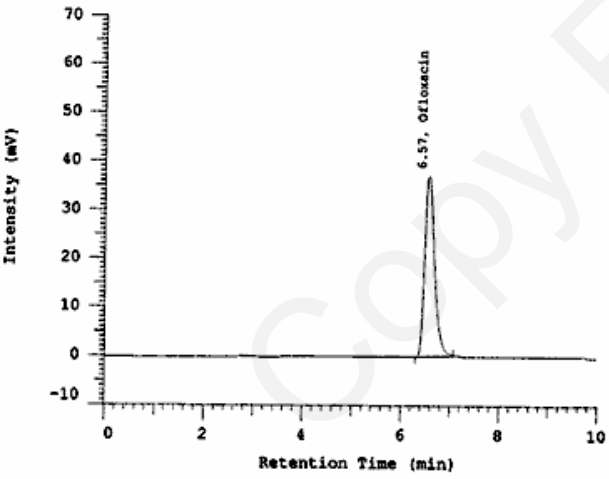
Assay of Tablet Formulation

The method was used for determination of ofloxacin and ornidazole in tablet formulations. The results obtained (Table-5) showed percentage recoveries were high and RSD (%) values were low, which confirms the method is suitable for routine determination of these components in their pharmaceutical preparations. Assay experiment results revealed that the content of ofloxacin

and ornidazole in tablets formulation was in the range of 101.09±0.40% and 100.51±0.64% respectively.

CONCLUSION

A new high performance liquid chromatographic method has been developed for the simultaneous determination of ofloxacin and ornidazole in tablet formulation. It was observed that the method is more sensitive, accurate, precise, repeatable with wide range of linear range as compared to the reported method [18,19]. The run time is relatively short i.e. less than 10 min, which enable rapid quantitation of samples in the routine analysis of tablet formulation. No interference has been observed from the tablet excipients. The method can be used for the simultaneous determination of ofloxacin and ornidazole in the tablet formulation.

	A
	B

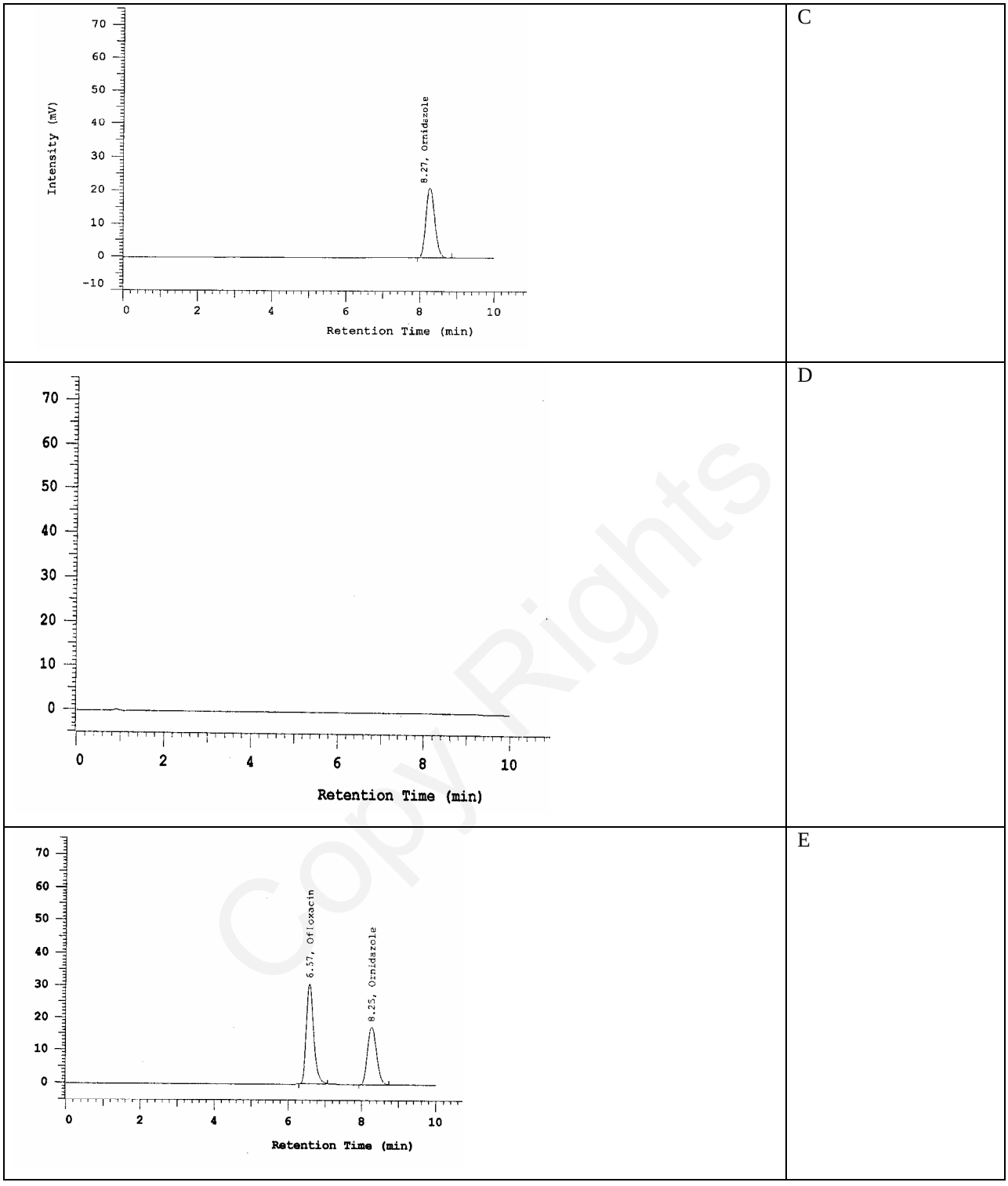


Figure 2: Chromatograms obtained from

A.) Ofloxacin (found 10.01 µg/ml) and ornidazole (found 25.2 µg/ml) sample solution

B.) Standard ofloxacin (12.00 µg/ml)

C.) Standard Ornidazole (30. 00 µg/ml)

D.) Placebo solution (obtained from starch, lactose, magnesium stearate, and Avicel)

E.) Standard ofloxacin (10.00 µg/ml) and ornidazole(25.00 µg/ml) solution mixture.

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تطوير وتقييم فاعلية التحليل المتزامن للدوائين الافلوكساسين والارنيدازول في الاقراص المنتجة باستخدام جهاز RP-HPLC

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ملخـص

تم تطوير طريقة خاصة بسيطة وحساسة بكلفة أقل باستخدام جهاز (HPLC) للقياس المتزامن لمادة الافلوكساسين و الارنيدازول في التركيب الكيميائي الصيدلاني ،وتمت عملية الفصل الكروماتوغرافي على عمود فصل راتنجي من نوع (BDS hypersil – C18) ذي الأبعاد التالية (250 مم للطول و4.6 مم للقطر الداخلي و10 مايكروميتر لقطر الجسيمات) وتم استخدام الطور الناقل المتكون من 80 % ماء (والمحتوي على 0.55 مليلتر لكل لتر من مادة ثلاثي ايثيل أمين لتحسين شكل الإشارة التحليلية) و20 % اسيتونايترايل وتم تعديل مقياس الاسس الهيدروجيني الى 3.0 باستخدام حامض الفسفوريك، وتم الكشف باستخدام طول موجة امتصاص 284 نانومتر وكانت الاستجابة بالدلالة الخطية للتركيز بمعدل 1-20 ميكروجرام لكل مليلتر للافلوكساسين و 2.5 ـــــــــ 50 ميكروجرام لكل مليليتر للارنيدازول، وكانت معاملات الارتباط 0.9998 و 0.9995 على التوالي، ومدى الكشف من 0.01 الى 0.02 ميكروجرام لكل مليليتر من الافلوكساسين والارنيدازول على التوالي بينما وجد حد الكميات من 0.05 الى 0.1 ميكروجرام لكل مليلتر وكانت صحة النتيجة لكل من الافلوكساسين والارنيدازول عند (80%) ، و(100%) ، و (120%) للدواء تتراوح بين(99.6 -100.9) وكانت الدقة خلال اليوم وبين الأيام اقل من 1 % وكان وقت التمليص الكلي للدوائين اقل من تسع دقائق .

الكلمات الدالة : الافلوكساسين، الارنيدازول، فعالية طريقة التحليل.

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