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HPLC Analysis for Norfloxacin and Tinidazole standard drug

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Abstract:

A simple, precise, accurate, robust and selective reversed phase high pressure liquid chromatographic method has been developed and validated for the "Simultaneous estimation of Norfloxacin and Tinidazole in bulk and dosage forms by UV and RP-HPLC and their cross validation". Phenomenex Luna $C_{18,}$ 5 µm, 250 × 4.6 mm I.D column with mobile phase 0.025 M Potassium Dihydrogen Phosphate buffer solution: Acetonitrile (40:60 v/v), flow rate 0.5 ml/min and the effluent monitored by UV detection at 290nm. The proposed method was validated for parameters viz., specificity, accuracy, precision, linearity, limit of detection, limit of Quantitation, robustness and system suitability, as per ICH guidelines for Norfloxacin and Tinidazole standard drug. The (Regression coefficient) r^2 value for linearity of Norfloxacin and Tinidazole was 0.997 and 0.998. The percentage recovery of Norfloxacin and Tinidazole was within the range of 95-105%.
The (Regression coefficient) r^2 value for linearity by UV of Norfloxacin and Tinidazole was 0.999 and 0.998. The percentage recovery of Norfloxacin and Tinidazole was within the range of 95-105%.

Key words: RP-LC Method, and UV Estimation, Norfloxacin, and Tinidazole

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1. Introduction

The cleanliness and hygiene is one of the major concern in developing country. In every year a huge portion of the people in India suffers from different kind diseases related to protozoa and bacteria. Due to unhygienic environment protozoal and bacterial infections continue to cause significant morbidity and mortality throughout India.[1]
Single antiprotozoal drug, sometimes shows its ineffectiveness for the eradication of protozoa from human body, so the physician prefers the combination of antiprotozoal drug.
The combination of norfloxacin and tinidazole is commercially available in tablet form to control various gastrointestinal infections caused by bacteria or ameobic infection, prostasis and urinary tract infections due to susceptible uropathogens.[2]
The literature review finds that the assay of this combination drug are not well validated by simple UV spectrophotometer and HPLC-UV method. Most of the HPLC-UV analysis executed by the previous researchers are costly time consuming due to their long run time. For this reason it is proposed to analyze these combination drugs simultaneously by well developed simple UV spectrophotometer and HPLC-UV method with reasonable shorter run time.[3].
So the need of our present study is to provide a simple, sensitive, precise and accurate validated method of HPLC analysis which will reduce the run cost of routine analysis.[4],[5].

2. MATERIALS:

- ✓ Volumetric flasks 10ml (Merk specialities private Ltd., Mumbai).
- ✓ Pipettes 1ml(Merk specialities private Ltd., Mumbai).
- ✓ HPLC grade Acetonitrile (Merk specialities private Ltd., Mumbai).
- ✓ HPLC grade water (Merk specialities private Ltd., Mumbai) & (Karnataka fine chem, Bangalore).
- ✓ HPLC grade Potassium Dihydrogen phosphate buffer (Finar chemicals Ltd., Ahmedabad).
- ✓ Norfloxacin bulk drug was provided by Acharya and B.M Reddy college of Pharmacy, Bangalore.
- ✓ Whatmann's filter paper.

- ✓ NAOH (Merk specialities private Ltd., Mumbai).

3. Results and Discussions:

Tinidazole and Norfloxacin: [6]
Tinidazole and Norfloxacin combination have been introduced into the market for their Anti-Protozoal drug bacterial infections. It is available in market Tinidazole and Norfloxacin in ratio 6:4. A thorough literature survey revealed that there is no suitable method available for the simultaneous estimation. A reversed phase HPLC method have been developed using Acetonitrile, and Phosphate buffer in ratio of 60:40 v/v. The system suitability parameters were found to be satisfactory. The Retention time for Tinidazole and Norfloxacin were at 6.623 and 4.224 retention time. The method was validated as ICH guidelines. The specificity studies proved that there is no interference from the excipients and other impurities. Linearity was found between 4 – 40 µg/ml for Norfloxacin and 6 – 60 µg/ml for Tinidazole with regression coefficient 0.997 and 0.998 respectively. LOD and LOQ values are 0.1µg/ml, 0.3 µg/ml for Norfloxacin and 0.1 µg/ml, 1.0 µg/ml for Tinidazole. All the parameters were within the acceptance criteria.[7]
Tinidazole and Norfloxacin:

Tinidazole – Norfloxacin combination claims to have Anti-Protozoal drug. It is available in the ratio 6:4. As the literature reports some spectrophotometric and LC method for the determination. Simultaneous equation method have been developed and successfully employed for the estimation in finished formulation. The percentage of contents obtained are 98.14% for Tinidazole and 98.50 % for Norfloxacin. To carry out the accuracy studies an RP-HPLC method was developed and percentage of contents are 99.01% for Tinidazole and 99.06 % for Norfloxacin. This method is simple, economic and readily used for the estimation of the combination in a short time.[8],[9].

Fig: 1 Norfloxacin linearity UV

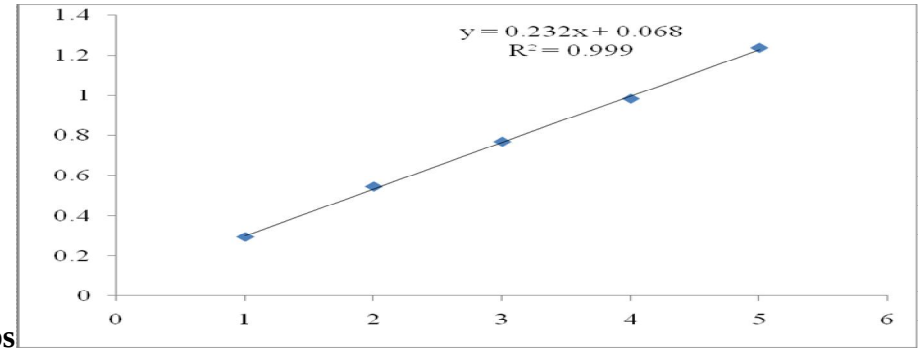
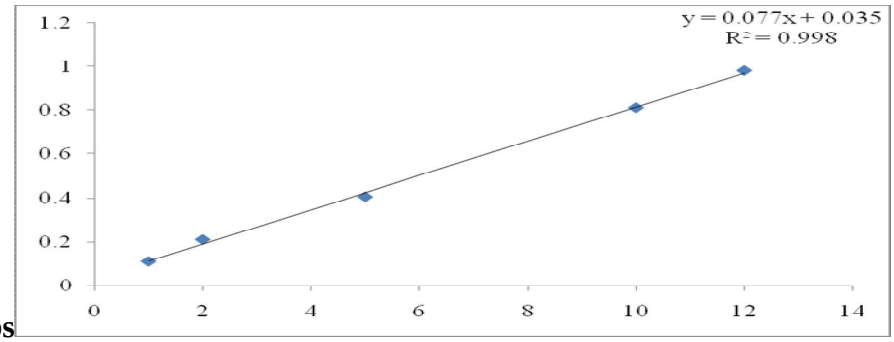


Fig: 2 Tinidazole Linearity UV



Abs

Conc µg/ml

Table no: 3 Linearity data for Tinidazole and Norfloxacin by UV

Conc (µg/ml)Tinidazole	Abs	Conc (µg/ml)Norfloxacin	Abs
1	0.1102	1	0.2949
2	0.2105	2	0.5446
5	0.4052	3	0.7673
10	0.8102	4	0.9826
12	0.9802	5	1.2363

Accuracy:

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy may often express as percentage recovery by the assay of known added amounts of analyte. The Percentage Recovery was found within Limit of 95-105% ,[10]

Table no: 4 Recovery studies for Tinidazole UV

S.N0	Initial drug concentration (µg/ml)	Standard drug added (µg/ml)	% Drug addition	Total Concentration		% Recovery
				Present	detected (µg)	

1	6	3	50%	9	8.84	98.26
2	6	6	100%	12	11.82	98.56
3	6	9	150%	15	14.79	98.63

Table no: 5 Recovery studies of Norfloxacin by UV

S.N0	Initial drug concentration (µg/ml)	Standard drug added (µg/ml)	% Drug addition	Total Concentration		% Recovery
				Present	detected (µg/ml)	
1	4	2	50%	6	7.86	98.30
2	4	4	100%	8	9.80	98.02
3	4	6	150%	10	11.80	98.35

Precision:

Precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample. Precision of an analytical method is usually expressed as standard deviation or relative standard deviation. [11]

Intraday precision- The repeatability of the method was assessed by Sampling 3 different Concentration samples of 3 replicates on single day

Table no: 6 Intraday precision Tinidazole by UV

Concentration (µg/ml)	Absorbance (Mean of 3 values)	SD	%RSD	Acceptance criteria
2	0.2105	0.0004	0.19	% RSD < 2
5	0.4052	0.0001	0.03	
10	0.8104	0.0002	0.02	

Table no: 7 Interday precision Tinidazole by UV

Concentration (µg/ml)	Absorbance (Mean of 3 values)	SD	%RSD	Acceptance criteria
2	0.2105	0.0002	0.09	% RSD < 2
5	0.4052	0.0003	0.07	
10	0.8105	0.0004	0.04	

Table no: 8 Intraday precision Norfloxacin by UV

Concentration (µg/ml)	Absorbance (Mean of 3 values)	SD	%RSD	Acceptance criteria
2	0.5444	0.0002	0.036	% RSD < 2
3	0.7671	0.0001	0.013	
4	0.9823	0.0002	0.02	

Table no: 9 Interday precision Norfloxacin by UV

Concentration (µg/ml)	Absorbance (Mean of 3 values)	SD	%RSD	Acceptance criteria
2	0.5444	0.0004	0.07	% RSD < 2
3	0.7674	0.0003	0.03	
4	0.9824	0.0002	0.02	

Table no: 10 Assay of Norfloxacin by UV

Drug	Label claim	Amount found	% Purity

Norfloxacin	**400mg**	**392.56mg**	**98.14%**

Table no: 11 Assay of Tinidazole by UV

Drug	**Label claim**	**Amount found**	**% Purity**
Tinidazole	**600mg**	**591mg**	**98.50%**

Validation:

Validation of an analytical method is the process to establish by laboratory studies that the performance characteristic of the method meets the requirements for the intended analytical application. Performance characteristics are expressed in terms of analytical parameters.[12]

Design of experiment:

The various analytical parameters used in analytical method validation are:

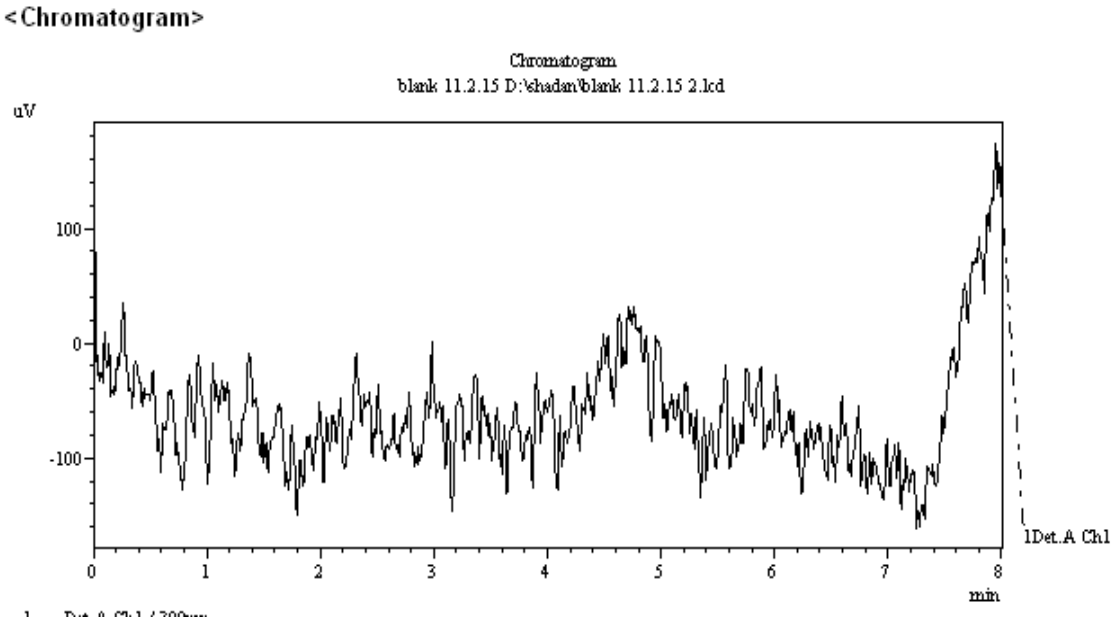
- Specificity
- Linearity and Range
- Precision – (Intraday, Interday)
- Accuracy
- Limit of Detection (LOD)
- Limit of Quantitation (LOQ)
- Robustness
- System suitability

SD – Standard Deviation

RSD – Relative Standard Deviation

Specificity:

Fig: 3. Chromatogram for specificity of the proposed method.



Chromatogram of blank no peaks at the retention time of Norfloxacin and Tinidazole.

This indicates that excipients used in the formulation do not interfere in the estimation of Norfloxacin and Tinidazole.

Linearity:

The linearity of analytical method is its ability to elicit test results which are directly proportional to the concentration of the analyte in the sample. A portion of 0.4, 0.8, 1.2, 2.0, and 4.0ml from 100 µg/ml of standard stock solution of Norfloxacin and a portion of 0.6,1.2,1.8,3.0 and 6.0ml from 100 µg/ml of standard stock solution of Tinidazole were transferred separately in to series of 10ml volumetric flask and diluted with mobile phase to get concentration of 4-40 µg/ml and 6-60 µg/ml Norfloxacin and Tinidazole. [12], [13]

Fig: 4

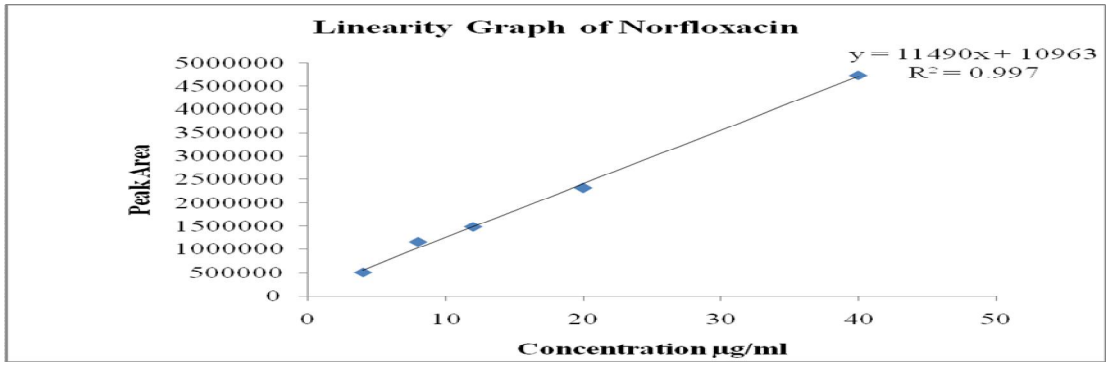
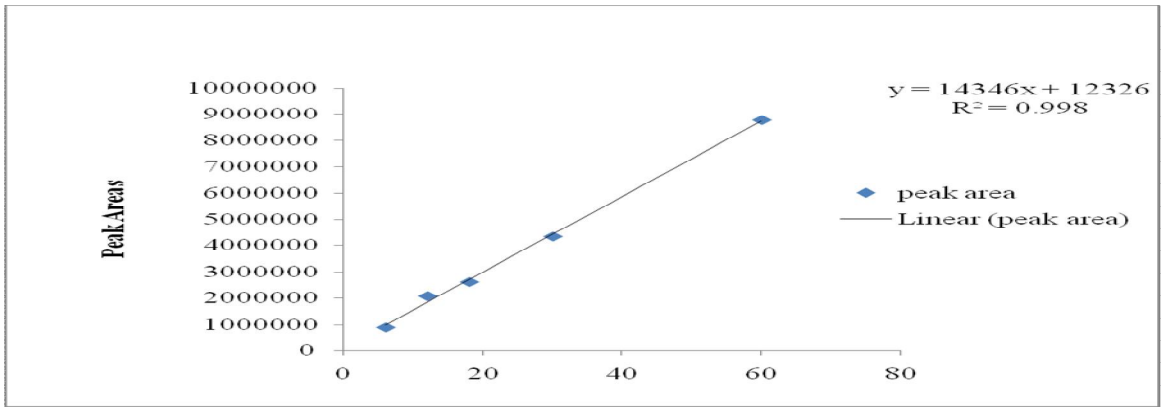


Table no : 12 Linearity data for Norfloxacin and Tinidazole

conc (µg/ml) Norfloxacin	peak area	conc (µg/ml) Tinidazole	peak area
4	510287	6	899004
8	1157288	12	2073082
12	1480660	18	2617052
20	2319494	30	4339466
40	4732637	60	8764807

Fig :5 Linearity data for

Tinidazole



Precision:

Precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample. Precision of an analytical method is usually expressed as standard deviation or relative standard deviation.

Intraday precision- The repeatability of the method was assessed by injecting 2 different ratio samples of 3 replicates on single day.[14]

Table no: 13 Intraday precision Tinidazole by HPLC

Concentration (µg/ml)	Peak area (Mean of 3 values)	SD	%RSD	Acceptance criteria
6	895080.66	9801.27	1.09	% RSD < 2
12	2063407.33	12651.87	0.47	
18	2626044.33	25707.65	0.97	

Interday precision- The repeatability of the method was assessed by injecting 2 different ratio samples of 3 replicates each on 3 different days

Table no: 14 Interday precision Tinidazole by HPLC

Concentration (µg/ml)	Peak area (Mean of 3 values)	SD	%RSD	Acceptance criteria
6	801400.66	8998.91	1.12	% RSD < 2
12	1108946	1317.65	0.16	
18	1833790.66	9457.91	0.51	

Table No: 15 Intra-day variability of Norfloxacin Standard drug

Concentration (µg/ml)	Peak area (Mean of 3 values)	SD	%RSD	Acceptance criteria
4	510380.33	468.89	0.09	% RSD < 2

8	1160051.33	5592.85	0.48	
12	1471023.33	9256.83	0.62	

Table No: 16 Inter-day variability of Norfloxacin Standard drug

Concentration (µg/ml)	Peak area (Mean of 3 values)	SD	%RSD	Acceptance criteria
4	395390.66	1548.55	0.35%	% RSD < 2
8	1058147.33	11827.85	1.11%	
12	6481402.66	95352	1.47%	

Table No: 17 Recovery studies of Norfloxacin by HPLC

S.N0	Initial drug concentration (µg)	Standard drug added (ml)	% Drug addition	Total Concentration		% Recovery
				Present	detected (µg)	
1	4	2	50%	6	7.85	98.38 %
2	4	4	100%	8	9.97	99.78 %
3	4	6	150%	10	11.87	98.99 %

Table No: 18 Recovery studies of Tinidazole by HPLC

S.N0	Initial drug concentration (µg)	Standard drug added (ml)	% Drug addition	Total Concentration		% Recovery
				Present	detected (µg)	
1	6	3	50%	9	8.93	99.38%
2	6	6	100%	12	11.92	99.34%
3	6	9	150%	15	14.89	99.32 %

Acceptance Criteria – 95-105% as per ICH Guideline.

Limit of detection (LOD):

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated as an exact value, under the stated experimental conditions.

The signal to noise ratio (S/N) of LOD is 3:1 or calculated by using the following formula

$$\mathbf{LOD = 3.3 \times \sigma / S}$$

Where,

σ = Standard deviation.

S = Slope.

Limit of Quantitation: (LOQ)

The Quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

The signal to noise ratio (S/N) of LOQ is 10:1 or calculated by using the following equation

$$\mathbf{LOQ = 10 \times \sigma / S}$$

Where,

σ = Standard deviation.

S = Slope. [15]

Table No: 19

Parameters	HPLC	
	NF	TZ
LOD	0.1µg/ml	0.1µg/ml
LOQ	0.3µg/ml	1µg/ml

Report: The LOD value of NOR and TZ are found to be 0.1µg/ml and LOQ values 0.3µg/ml and 1.0µg/ml for NOR and TZ indicating respectively, indicating good sensitivity of system and method.

Fig 10.

<Chromatogram>

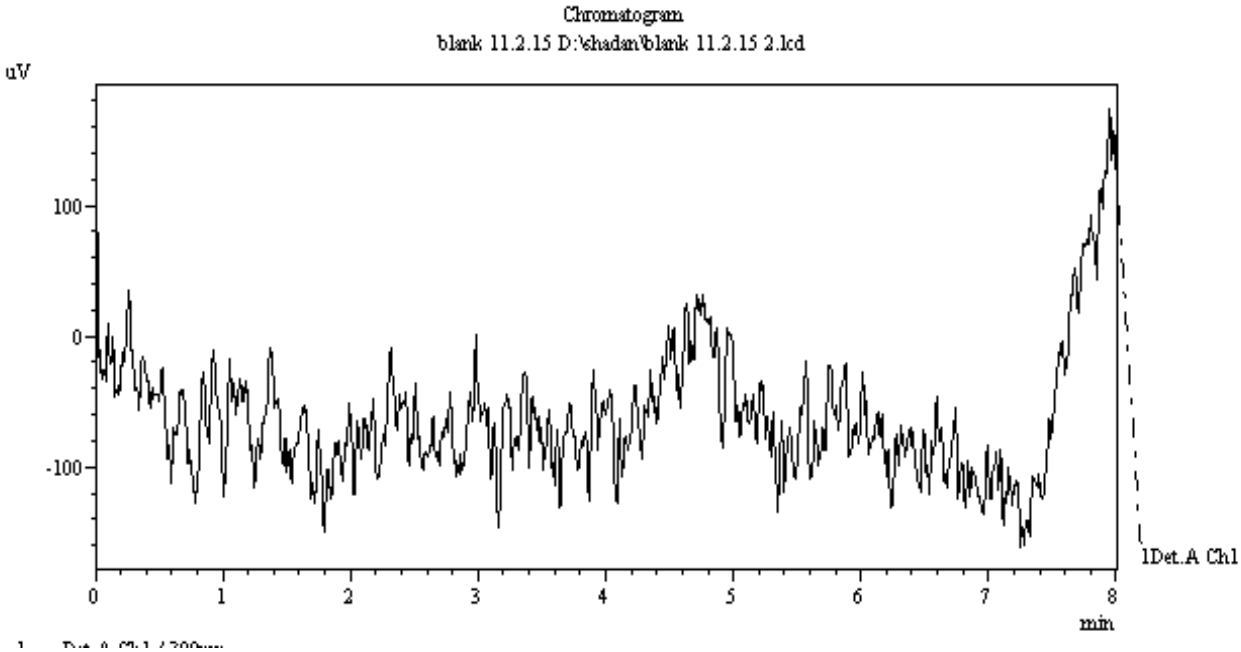


Fig 11. LOD NORFLOXACIN 0.1 µg/ml.

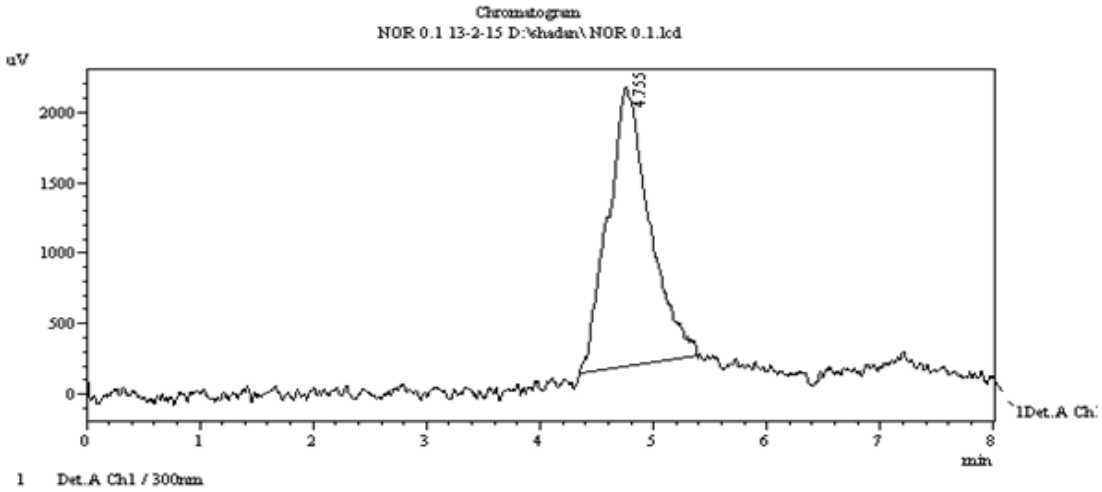


Fig 12. LOQ NORFLOXACIN 0.3 µg/ml.

<Chromatogram>

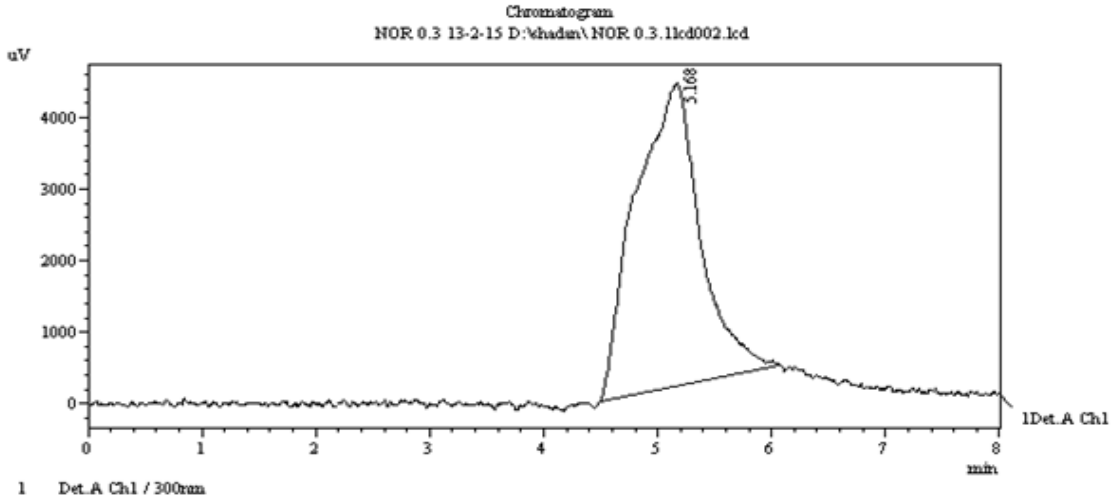


Fig 13. LOD TINIDAZOLE 0.1 µg/ml.

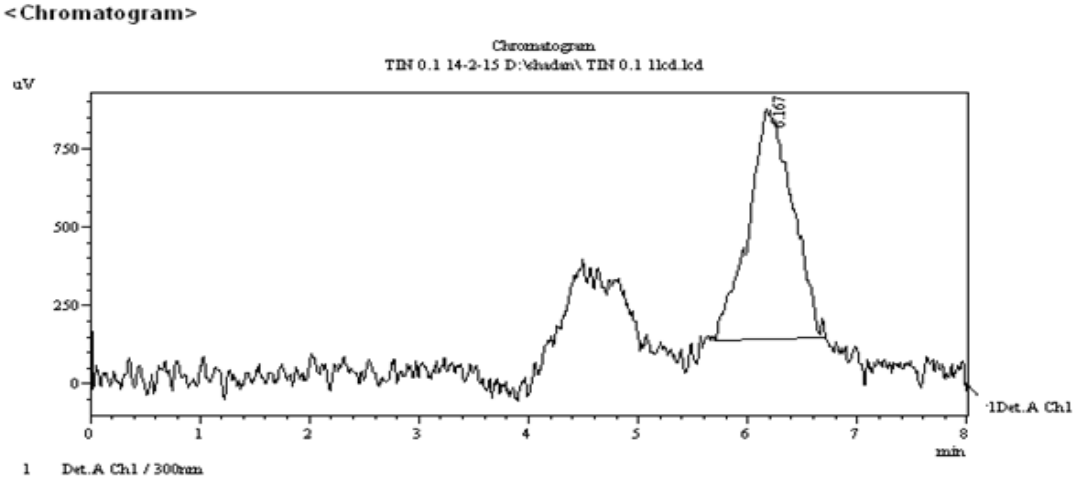
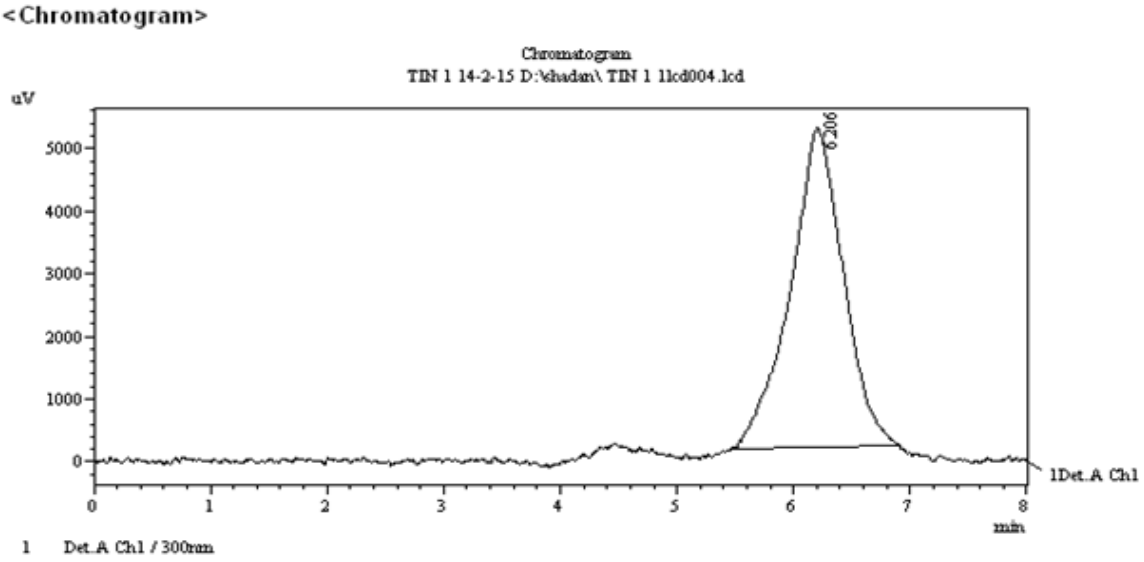


Fig 14. LOQ TINIDAZOLE 1 µg/ml.



Robustness:

Table No: 20 Variations in flow rate for Norfloxacin and Tinidazole standard drug

Flow rate (ml/min)	t_R (min)	Change in t_R (min)	t_R (min)	Change in t_R (min)	Acceptance criteria
	Norfloxacin		Tinidazole		% RSD < 1

0.48.	4.934	0.125	6.332	0.281
0.50	**4.809**		**6.613**	**-**
0.52	4.563	0.246	6.034	-0.579

Table No: 21 variations in mobile phase composition for Norfloxacin and Tinidazole standard drug

Acetonitrile: Phosphate buffer 60:40 (v/v)	**t_R (min)**	**(mi Change in t_R n)**	**t_R (min)**	**Change in t_R (min)**	**Acceptance criteria**
	Tinidazole		**Norfloxacin**	**Norfloxacin**	
62:38	6.032	0.581	4.828	0.019	**% RSD < 1**
60:40	**6.613**		**4.809**	**-**	
58:42	6.158	0.455	4.734	0.075	

Table No : 22 Variations in wavelength of Norfloxacin and Tinidazole standard drug.

Wave length (nm)	**t_R (min)**	**Change in t_R (min)**	**t_R (min)**	**Change in t_R (min)**	**Acceptance criteria**
	Tinidazole		**Norfloxacin**		
302	6.256	0.357	4.725	-0.084	**% RSD < 1**
300	**6.613**		**4.809**	**-**	
298	6.260	0.353	4.731	+0.078	

System suitability:

System suitability is checked to assess the developed chromatographic conditions which are suited for the analysis. It is further substantiated with accuracy and precision results.

A portion of 0.6ml of standard stock solution of Tinidazole, 0.4 ml of Norfloxacin is mixed in to 10 ml volumetric flask from 100 µg/ml stock solution. It was suitably diluted with mobile phase to get a required concentration. Resulting solution was sonicated for 10 minutes and 20µl of this standard solution was injected into the HPLC system.

System suitability parameters are given in table 23.

Table No: 23 System suitability

SL. NO.	PARAMETE R	BULK DRUG	BULK DRUG	ACCEPTANCE CRITERIA
		Norfloxacin	Tinidazole	
1.	Theoretical plates	2483.81	4571.67	n>2000
2.	Tailing factor	1.711	1.438	n<2

Table No: 24 Assay of Norfloxacin Tablet 100 µg

Drug	Label claim	Amount found	% Purity
Norfloxacin	**400mg**	**396.06mg**	**99.01%**

Table No: 25 Assay of Tinidazole Tablet 100µg - (187107)

Drug	Label claim	Amount found	% Purity
Tinidazole	600mg	594.36mg	99.06%

Cross Validation: Method validation was performed with respect to linearity, Precision and accuracy in order to evaluate the reliability of the results. The obtained validation results are summarized.

Table No:26

	Linearity			ACCURACY			PRECISION		
	slope	Intercept	Regression coefficient						
RP-HPLC				Low Qc	Middle	High	Low Qc	Middle	High
NOR	11490	10963	0.997	98.38	99.78	98.99	0.09	0.48	0.62
TIN	14346	12326	0.998	99.38	99.34	99.32	1.09	0.47	0.97
UV NOR	0.232	0.068	0.999	98.30	98.02	98.35	0.19	0.03	0.02

TIN	**0.077**	**0.035**	**0.998**	**98.26**	**98.56**	**98.63**	**0.03**	**0.01**	**0.02**
Interday precision (HPLC)									
NOR(HPLC)							**0.35**	**1.11**	**1.47**
TIN(HPLC)							**1.12**	**0.16**	**0.51**
NOR (UV)							**0.07**	**0.03**	**0.02**
TIN(UV)							**0.09**	**0.07**	**0.04**

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