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NEW RP- HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF TINIDAZOLE AND CIPROFLOXACIN AND ETHYLENEDIAMINE IMPURITIES IN TABLET DOSAGE FORM

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ABSTRACT

A Simple, precise and accurate method was developed for the estimation of Tinidazole, Ciprofloxacin and related impurities (ethylenediamine) analysis.For RP-HPLC method Mobile phase – A is pH 4.5 KH_2PO_4 buffer and mobile phase-B is P^H 4.5 Potassium dihydrogen phosphate buffer and Acetonitrile in the ratio of 50:50%v/v was selected as a mobile phase gave retention times 21.93, 11.02 and 18.47 respectively for Ciprofloxacin, Tinidazole and Ethylenediamine. The column used was X-Terra C18 column, 150 × 4.5mm i.d, 5µm with flow rate 1ml/min using UV detection at 278 nm &317nm. The correlation coefficient of Ciprofloxacin, Tinidazole and Ethylenediamine was found to be 0.999 **.**The limit of quantification for Ciprofloxacin, Tinidazole and Ethylenediamine was found to be 0.1050µg/ml, 0.1530µg/ml and 0.1190 µg/ml respectively. The accuracy was found to be within the limits. The precision was with in the acceptance criteria not more than 15.0% for each individual impurity. Hence it is conclude the developed RP-HPLC method can be effectively used for estimation of Ciprofloxacin, Tinidazole and and their related impurities from pharmaceutical dosage forms.

Keywords: RP-HPLC, Tinidazole, Ciprofloxacin, Ethylenediamine.

INTRODUCTION

Tinidazole chemically 1-(2-ethylsulfonylethyl)-2-methyl-5-nitro-imidazole.The empirical formula is $C_8H_{13}N_3O_4S$ and molecular weight is 247.273 g/mol. It is a synthetic nitroimidazole derivatives used as an antiprotozoal, antibacterial agent, chemically reduced tinidazole was shown to release nitrites and cause damage to purified bacterial DNA in vitro. Additionally, the drug caused DNA base changes in bacterial cells and DNA strand breakage in mammalian cells. It absorbs rapidly and completely under fasting conditions. Oral absorption of tinidazole is found to be 100%[10]. Ciprofloxacin chemically1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1, 4- dihydroquinoline-3-carboxylic acid and empirical formula is $C_{17}H_{18}FN_3O_3$ and molecular weight is 331.4.

It is used as an antibacterial agent, these antibiotic drugs inhibit the bacterial DNA gyrase enzyme which is necessary for DNA replication. Since a copy of DNA must be made each time a cell divides, interfering with replication makes it difficult for bacteria to multiply[6]. Ciprofloxacin is available for oral administration in 250mg, 500mg, 750mg and 1000mg tablets, Parental administration in 2mg/ml, Ophthalmic solution in 0.3% as base. Rapidly and well absorbed from gastro intestinal tract undergoes minimal first pass metabolism. The

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absolute bioavailability of ciprofloxacin tablets 50-85% after oral administration [1].

The aim of study was to develop a simple, accurate, precise RP-HPLC method for estimate tinidazole and ciprofloxacin and related substance analysis in tablet dosage form .

MATERIALS AND METHODS

Drug Samples:

Tinidazole and Ciprofloxacin, Related impurities materials were obtained from PVS group of Pharmaceuticals, vijayawada.

Chemicals:

Potassium dihydrogen phosphate : Merck
Orthophosphoric Acid : Merk
Water : Milli-Q
Methonal : Merk
Acetonitrile : Rankem

Instruments Used:

HPLC : Equipped with a photodiode array detector capable operating in the range of 190 nm to 400 nm - Waters
pH meter : Range from 0-14 - Sartorius
Analytical Balance : Accurate to 0.1 µg - Sartorius
Graduated Cylinder : 50 mL, 100 mL, and 1000 mL - Borsil,
Volumetric Flasks : 500 mL, 100 mL, 50 mL, 10 mL, 5 mL - Borsil,
Volumetric Pipettes : 10 mL, 5 mL, 3 mL, 2 mL, 1 mL – Borsil,
Graduated Pipettes : 10 mL, 5 mL, 2 mL, 1 mL - Borsil,
Solvent filtration unit : Millipore – Rankem
Syringe Filters : PVDF filters - Zodiac Life Sciences
Water bath : Lab companion
Sonicator : Sonorex

METHOD DEVELOPMENT

Optimized Chromatographic Conditions

Column : X-Terra, RP-8 (250 mm x 4.6 mm), 5µm
Buffer preparation : Weigh accurately 3.4 g of in 1000 mL Milli-Q water and add 5 mL of Triethylamine and mix with glass rod to dissolve
KH_2PO_4 Buffer : KH_2PO_4 buffer pH to 4.5 with ortho phosphoric acid
Organic solvents : Acetonitrile, Methanol
Mobile phase - A : pH 4.5 KH_2PO_4 buffer
Mobile phase - B : KH_2PO_4 buffer: Acetonitrile and pH 4.5 (Ratio 50:50)
Diluent : KH_2PO_4 buffer: Acetonitrile and pH 4.5 (Ratio 70:30)
Sonication time : More than 10 min to degas the mobile phase
Mode : Gradient
Flow Rate : 1.0 mL/min
Column Temperature : $25^0c \pm 2^0c$
Injection Volume : 10µL
Run Time : 65 min
Needle Wash : Methanol
Detector : HPLC equipped with 278nm for Ciprofloxacin & 317nm for Tinidazole UV detector.

Gradient program:

Time	MP-A%	MP- B %
0.01min	90.0	10
30.00 min	70.0	30
45.00min	40.0	60
53.00 min	40.0	60
55.00 min	90.0	10
65.00 min	90.0	10

Relative retentions times for impurities:

Impurity Name	**Relative Retention Time**
Ethylenediamine :	18.292
Compound Name	**Retention Time**
Ciprofloxacin :	21.780
Tinidazole :	10.891

RRT's of the Impurity are calculated against Ciprofloxacin peak.(Fig .1)

Standards used:

Ciprofloxacin Hcl Working Standard of known Potency: 93.19%
Tinidazole Working Standard of known Potency: 99.69 %

Reagent Solutions Preparation

Preparation of pH 4.5 buffer:

Weigh accurately 3.4 g of KH_2PO_4 in 1000 mL Milli-Q water and add 5 mL of Triethylamine and mix with glass rod to dissolve KH_2PO_4 Adjust pH to 4.5 with Ortho Phosphoric acid. Filter the above solution through 0.45µm PVDF filter or equivalent.

Mobile Phase

Mobile phase-A: pH to 4.5 buffer and degassing is done about 10 min in sonicator.

Mobile phase-B: Mix Acetonitrile and pH To 4.5 buffers in the ratio of 50:50v/v respectively and degassing is done about 10 min in sonicator [1]

Preparation of Diluent

Mix pH 4.5 Buffer and Acetonitrile in the ratio of 50:50 v/v respectively. Degas in a Sonicator for about 10 minutes.

Preparation of Resolution Solution:

Impurity Solution Preparation:

Transfer about 5 mg of Ciprofloxacin Impurity C CRS (ethylenediamine analog compound) or working standard, accurately weighed to a 50 mL volumetric flask add about 25ml of diluent, mix well and made up to volume with diluent. Transfer 5.0ml of the above solution into a 20ml volumetric flask, dilute to volume with diluent and mix.

Transfer about 25mg of Ciprofloxacin Hydrochloride CRS or working standard accurately weighed to a 100 mL volumetric flask. Add about 50ml of diluent to dissolve.Add 5.0 ml of above diluted impurity solution. Dissolve and dilute to volume with diluent and mixed (Fig.5)

Preparation of Standard

Transfer the amount equivalent to about 25mg of Ciprofloxacin (without Tinidazole) working standard

Accurately weighed to a 100 mL volumetric flask. Add about 50mL of diluent and sonicate for 15 minutes, dilute to volume with diluent and mix. Filter about 2 mL through 0.45µm Nylon 66 membrane filter or equivalent .

Preparation of Placebo solution:

Accurately weighed,and transfer the placebo equivalent to about 25mg to a 100 mL volumetric flask. Add about 50mL of diluent and sonicate for 15 minutes, dilute to volume with diluent and mix. Filter about 2 mL through 0.45µm Nylon 66 membrane filter or equivalent.

Preparation of Test Solution

Crush not less than 20 tablets to a fine powder in a mortar with pestle. Transfer tablet powder equivalent to about 25 mg of Ciprofloxacin, and 5 mg of tinidazole separately weighed, to a 100 mL volumetric flask. Add about 50 mL of diluent and sonicate for 15 minutes, dilute to volume with diluent and mix. Filter about 2 mL through 0.45µm Durapore hydrophilic membrane filter or 0.45µm Pall Pharmalab Nylon 66 membrane filter .

Formula for Calculation of % impurity of Known Impurities

Process the chromatograms at 278nm & 317nm.

a) Known impurity at 278nm(ethylene diamine analog impurity)

% impurity

$$= \frac{TA_{EDA} \times WS_{EDA} \times 5 \times 5 \times 100 \times Avg\ wt \times P_{EDA} \times 100}{SA_{EDA} \times 50 \times 20 \times 100 \times W_{SAM} \times L.C \times 100}$$

TA_{EDA} = Peak area of Ethylenediamine analog in Sample chromatogram

SA_{EDA} = Average Peak area of Ethylenediamine analog in Resolution Chromatogram

WS_{EDA} = Weight of Ethylenediamine analog in Standard Chromatogram

W_{SAM} = Weight of Sample in test Preparation

P_{EDA} = Purity of Ethylenediamine analog impurity

W_{TS} = Weight of sample taken in mg for Sample preparation

L.C = Label claim of Ciprofloxacin

Unknown highest individual impurity

% of individual impurity = ri x 100/rt

1) Calculation of individual impurity to be done at nm which has more response.

2) Disregard the same at another nmReport the maximum individual impurity from the below[9]

Where,

ri = Highest area of individual unknown impurity other than blank and placebo at 278nm or 317 nm.

rt = Area of sum of all peaks, other than Blank, placebo and secondary principal peak for that particular nm[3]

(b) Total impurities

Sum of all individual impurities at 278 nm and 317 nm (Excluding Ethylenediamine analog impurity, Blank and Placebo)[3]

VALIDATION PARAMETERS

Linearity of detector response: To demonstrate the linearity of detector response for Ciprofloxacin, Tinidazole and its impurities prepare not less than six solutions with concentrations ranging from Limit of quantification level 50% to 200% of the target concentration at specification limit. The calibration curve was plotted using peak area ratio Vs concentration of the standard solution [4]. From the calibration curve, the slope and intercept were calculated (table 1(a),Table (b)). The correlation coefficient was found to be 0.999 (Fig.2,Fig.3,Fig.4).

System Suitability

The system suitability studies were carried out as specified in USP. These parameters include column efficiency, resolution, tailing factor, related standard deviation, number of theoretical plates, relative retention time and capacity factor.

The system suitability impurity solutions were prepared by using their related impurities of Ciprofloxacin and Tinidazole are injected into the HPLC system. The standard solution was prepared by using Ciprofloxacin and Tinidazole working standard as per test method and injected six times into the HPLC system.The summerised value given (table 2). The system suitability parameters were evaluated and found to be within the limits[4].

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value that is accepted either as a conventional true value or as an

accepted reference value and the value found. To determine the accuracy of the test method samples were prepared by homogeneous blend of tablet as per manufacturing formula or use the drug product, API and placebo can also be individually weighed and mix for each solution. Prepare stock solutions of all known impurities prepare sample solutions in triplicate (6-preperations for higher level) by spiking test preparation with impurity stock solutions at 50%, 75%, 100%, 125%, and 150% of specification limit to the target concentration. Calculate the % recovery of all the impurities. Tabulate the results in the table given below and accuracy at 150% level should be used in range (Table 4,5,6). Accuracy determination is done for every impurity while doing related substances validation to determine the amount of impurity found[7].

Precision

Precision of the test method by injecting six test samples prepared by spiking all the impurities at specification limit to the target concentration. And also precision study for diluted standard, by spiking working standard at specification level (maximum allowable % level for unknown impurity) on placebo. Inject the solutions in the chromatographic system as per the method then calculate the individual % Relative standard deviation of diluted standard and all the impurities (Table.3).

Limit of Detection (Lod) & Limit of Quantification (Loq)

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

Determine the limit of Quantification and limit of Detection Ciprofloxacin, Tinidazole and EDA impurities based on signal to noise ratio method. For the limit of detection of Ciprofloxacin, Tinidazole and EDA by identifying the concentration which gives a signal to noise ratio about 3. Determine the signal to noise ratio at analyte concentration of 0.005%. If signal to noise ratio is 10 or more, qualify this concentration as limit of quantification. If signal to noise ratio is less than 10, increase the concentration but not beyond 0.05%, to achieve the signal to noise ratio 10 and then qualify the concentration as limit of quantification[8].

Determine the Precision and Accuracy at Limit of quantification level. Prepare six test solutions by spiking the all the impurities, Ciprofloxacin, Tinidazole and EDA at about Limit of Quantification level in placebo equivalent to test concentration and inject into HPLC. If the drug product contains the known impurities, perform the study in the presence of placebo sample (Table.7,8,9,10). Calculate the % RSD for all the impurities, Ciprofloxacin, Tinidazole and EDA from six preparations [5].

The signal to noise ratio of Ciprofloxacin, Tinidazole and EDA was found to be 2.841, 2.944 and 2.938 at LOD level and 10.066, 9.405 and 10.781 at LOQ level respectively, hence the same was qualified as concentration for LOQ [4].

RESULT AND DISCUSSION

Linearity

Quantification level 50%, 200% of the target concentration at specification limit. The calibration curve was plotted using peak area ratio Vs concentration of the standard solution. From the calibration curve, the slope and intercept were calculated**.** Summarize the results in the table given below. The correlation coefficient was found to be 0.999. The results and graph are summarized and the results are summarized in table below.

Recovery

- Accuracy was performed in six times for higher levels and triplicate for remaining levels by spiking with the equivalent amount of sample at 50%, 75%, 100%, 125% and 150% of the target specification concentration into each individual volumetric flask.

The average % recovery was found to be within the limits. The % relative standard deviation recovery of Ciprofloxacin and Tinidazole 150% level was found to be within the limits.

Acceptance Criteria: The Correlation Coefficient for Ciprofloxacin, Tinidazole and its impurities should be not less than 0.997; Bias at 100% response should be not more than 5%.

Criteria: The results of the system suitability parameters should be within the specified limits.

Acceptance Criteria: The %RSD of Ciprofloxacin, Tinidazole and Ethylenediamine obtained from six preparations should not be more than 2%.

Acceptance Criteria: The method is considered accurate, if the individual recovery should be between 85.0% to 115.0%

Acceptance Criteria

1. Limit of detection: About 3 (2.0 – 3.4)
2. Limit of Quantification: About 10 (From 9.0 to 11.4).

Acceptance Criteria

The % Mean recovery of Ethylenediamine Analog, Ciprofloxacin and Tinidazole at limit of Quantification level should be between 85% to 115%.

Table 1 (a). Linearity test method

S.No	Ethylenediamine		Ciprofloxacin		Tinidazole	
	Conc. in 'µg/mL'	Peak Area	Conc. in 'µg/mL'	Peak Area	Conc. in 'µg/mL'	Peak Area
LOQ	0.125	7932	0.125	4260	0.150	3263
50%	0.625	36222	125.52	6971270	150.66	3269532
75%	0.937	56128	188.28	10644205	225.99	4795832
100%	1.250	78345	251.04	14250659	301.32	6400781
125%	1.500	96188	313.80	17953930	376.65	7906826
150%	1.875	124441	376.56	21714723	451.98	9461650
200%	2.501	163100	502.08	28457841	602.64	12379130

Table 1(b). Linearity test method

S.No	Parameter	Ciprofloxacin	Tinidazole	Ethylenediamine Analog
1	Correlation Coefficient (r)	0.999	0.999	0.999
2	Bias at 100% response	0.4	0.2	4.7
3	Intercept	62428	12476	3695
4	Slope	57149	20548	66719

Table 2. System Suitability

System suitability parameters	Observed value	Acceptance limit
The Resolution between Ciprofloxacin and ethylenediamine analog impurity	6.15	NLT 2.0
% RSD the of ethylenediamine analog	3.2	NMT 10.0

Table 3. Precision of Ciprofloxacin, Tinidazole and EDA Impurity

Sample Name	Peak Area		
	Ethylenediamine	Ciprofloxacin	Tinidazole
Sample 1	7334	4048	3381
Sample 2	7371	4015	3430
Sample 3	7358	4025	3440
Sample 4	7388	3982	3403
Sample 5	7358	4048	3425
Sample 6	7334	4063	3472
Avg	7357	4026	3425
%RSD	0.3	0.9	1.0

Table 4. Accuracy of Ciprofloxacin

S.No	Spike level	'µg/ml' added	'µg/ml' found	% Individual recovery
1.	150%	320.04	311.46	97.3
2.	150%	322.78	310.11	96.1
3.	150%	322.96	312.27	96.7
4.	150%	319.10	311.61	97.7
5.	150%	320.90	312.25	97.3
6.	150%	320.38	311.59	97.3

Table 5. Accuracy of Tinidazole

S.No	Spike level	'µg/ml' added	'µg/ml' found	% Individual recovery
1.	150%	449.03	452.03	100.7
2.	150%	454.50	451.04	99.2
3.	150%	452.12	453.54	100.3
4.	150%	451.42	452.32	100.2
5.	150%	452.71	453.06	100.1
6.	150%	453.11	452.63	99.9

Table 6. Accuracy of Ethylenediamine (EDA)

Sample No.	Spike level	'µg/mL' added	'µg/mL' found (recovered)	% recovery	Mean % recovery
1.	50%	0.6265	0.6175	98.6	98.9
2.	50%		0.6225	99.4	
3.	50%		0.6175	98.6	
1.	75%	0.9522	0.9375	98.5	97.9
2.	75%		0.9225	96.9	
3.	75%		0.9350	98.2	
1.	100%	1.2529	1.2375	98.8	98.4
2.	100%		1.2175	97.2	
3.	100%		1.2425	99.2	
1.	125%	1.5035	1.4825	98.6	98.0
2.	125%		1.4600	97.1	
3.	125%		1.4800	98.4	
1.	150%	1.8794	1.8500	98.4	98.0
2.	150%		1.8425	98.0	
3.	150%		1.8400	97.9	

Table 7. LOD of EDA, Ciprofloxacin and Tinidazole

Name of the Compound	LOD			LOQ		
	Ppm	%	S/N Ratio	ppm	%	S/N Ratio
Ciprofloxacin	0.010	0.004	2.841	0.105	0.042	10.066
Tinidazole	0.015	0.005	2.944	0.150	0.050	9.405
Ethylenediamine analog (EDA)	0.062	0.025	2.938	0.125	0.050	10.781

Table 8. Precision at LOQ levels of EDA

EDA	"µg/ml" added	"µg/ml" Found	% Individual Recovery	% Mean recovery
01	0.1250	0.1180	94.4	94.8
02	0.1250	0.1190	95.0	
03	0.1250	0.1190	94.9	

Table 9. Precision at LOQ level of ciprofloxacin

Ciprofloxacin	"µg/ml" added	"µg/ml" Found	% Individual Recovery	% Mean recovery
01	0.1054	0.1050	99.6	100.4
02	0.1054	0.1075	102.0	
03	0.1054	0.1050	99.6	

Table 10. Precision at LOQ level of Tinidazole

Tinidazole	"µg/ml" added	"µg/ml" Found	% Individual Recovery	% Mean recovery
01	0.1502	0.1500	99.9	101.9
02	0.1502	0.1560	103.9	
03	0.1502	0.1530	101.9	

Table 11. Summary Of Validation Parameters Of Ciprofloxacin, Tinidazole And EDA Impurities

Validation Parameters	Ciprofloxacin	Tinidazole	EDA	limit	Pass/fail
Linearity range (%)	LOQ-200 %	LOQ-200 %	LOQ-200 %	No limit	Passed
Correlation co-efficient (r^2)	0.999	0.999	0.999	NLT 0.997	Passed
LOD (PPM)	0.010	0.015	0.062	No limit	Passed
LOQ (µg/ml)	0.105	0.150	0.125	No limit	Passed
Precision RT (%RSD)*	0.9	1.0	0.3	NLT 15%	Passed
Accuracy at LOQ level %	99.6	99.9	97.8	85-115	Passed
Accuracy at 150 (%) level	97.3	100.1	98.0	85-115	Passed

Figure 1. Relative retension of tinidazole, ciprofloxacin, EDA

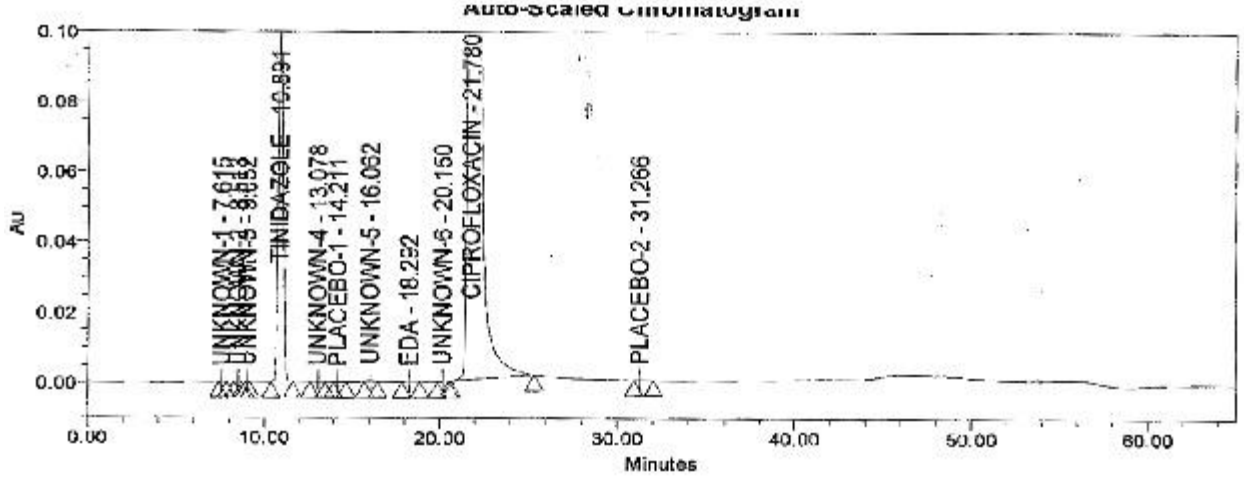


Figure 2. Linearity Of Test Method For Eda

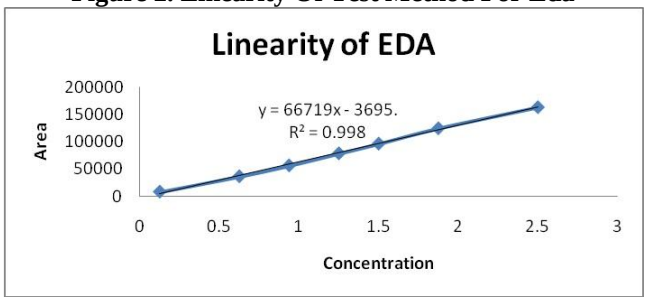


Figure 3. Linearity Of Test Method For Ciprofloxacin

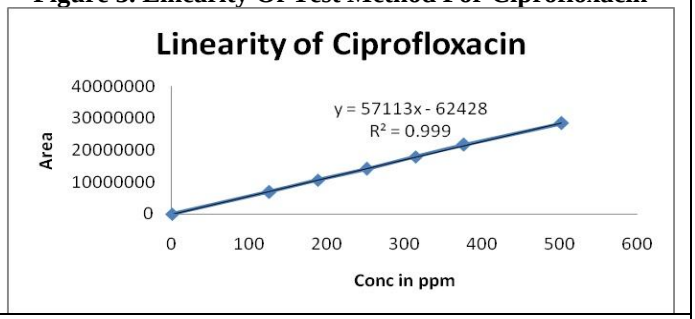


Figure 4. Linearity Of Test Method For Tinidazole

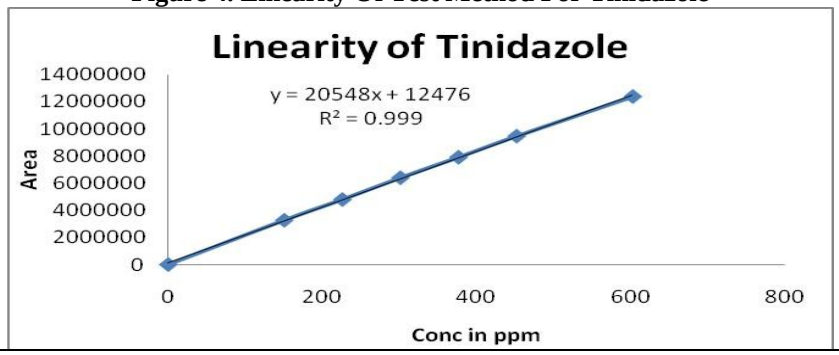


Figure 5. Resolution Chromotogram of EDA Against ciprofloxacin at 278 nm

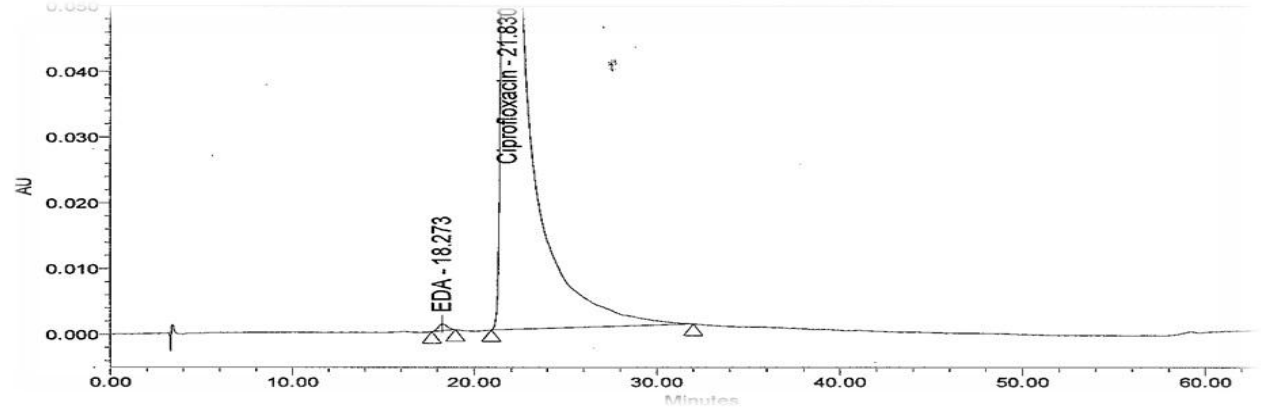


Figure 6. Typical Standered Chromotogram of ciprofloxacin at 278nm:

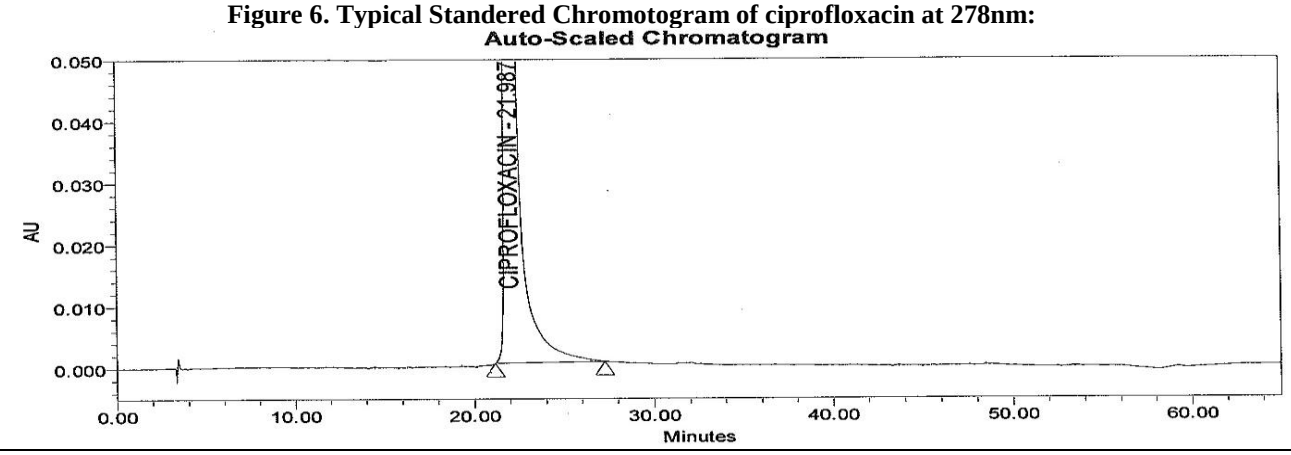


Figure 7. Typical Standered Chromotogram of Tinidazole at 317nm

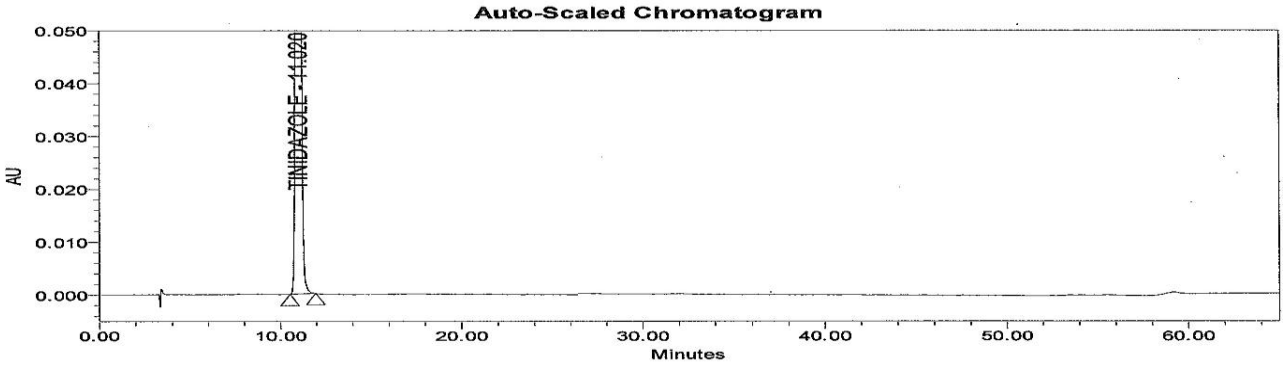


Figure 8. Typical sample chromatogram of ciprofloxacin,Tinidazole,EDA at 278nm

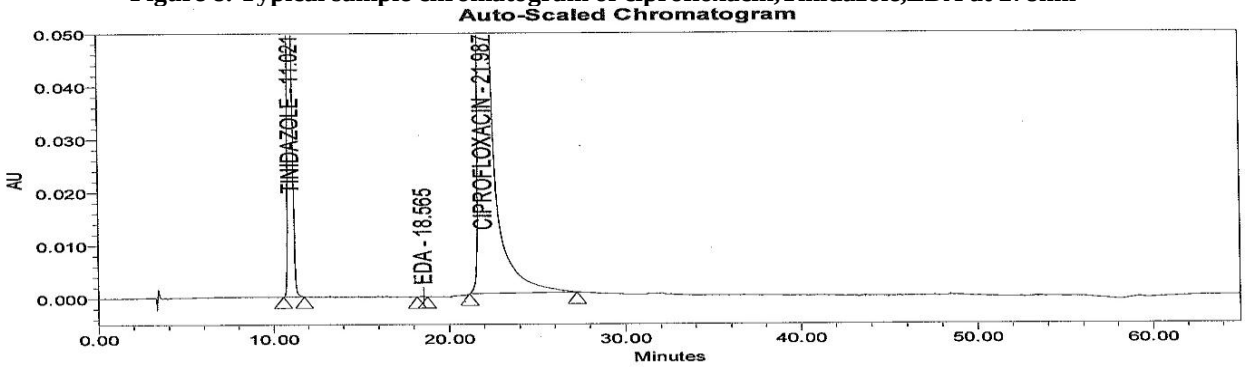
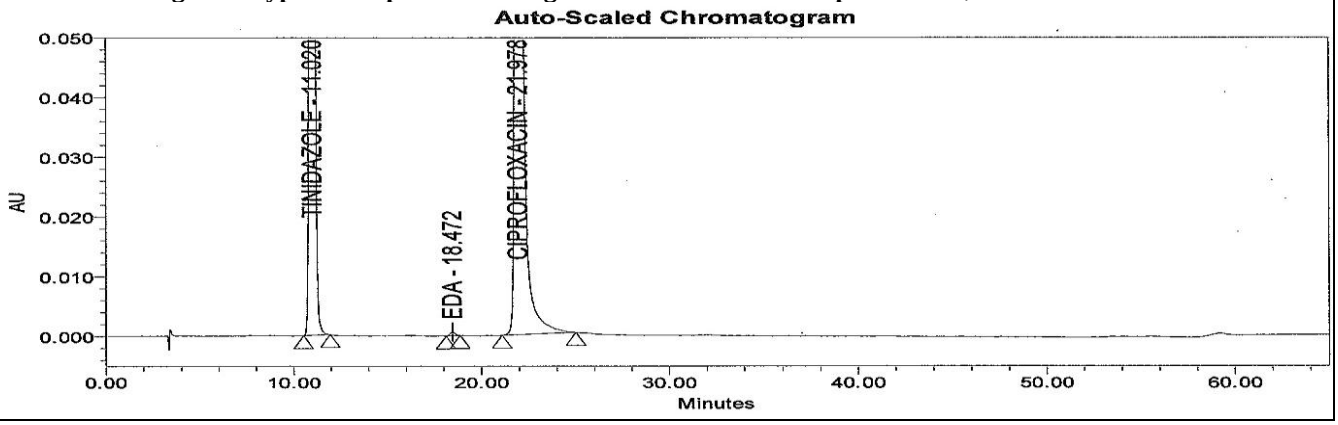


Figure 9. typical sample Chromotogram at Tinidazole and ciprofloxacin, EDA at 317nm



CONCLUSION

As per discussion in the Literature review, indicated that UV-Spectrophotometric, LC-MS, HPLC, RP-HPLC and HPTLC and individual and combination methods have been reported for determination of Ciprofloxacin and Tinidazole in pharmaceutical dosage form. An extensive literature survey reveals few methods are reported for estimation of related substances of Ciprofloxacin and Tinidazole in tablet formulation by RP-HPLC method. Most of the reported methods either do not include stress degradation studies or are not completely validated, and they are cumbersome, time consuming. Method validation is an essential step in drug analysis and for RP-HPLC method P^H 4.5 Potassium phosphate buffer as mobile phase –A and mixture of P^H 4.5 Potassium hydrogen phosphate buffer and Acetonitrile in the ratio of 50:50v/v was selected as a mobile phase-B which gives good resolution and good peak shapes for Ciprofloxacin, Tinidazole and their related substances and the primary objective of the present work to optimize condition to develop estimation of related substances (Ethylenediamine) of Ciprofloxacin and Tinidazole by RP-HPLC method. The flow rate was set at 1.0 ml/min. The correlation coefficient of Ciprofloxacin, Tinidazole and their related substances was found to be 0.999.

The developed method was validated according to ICH validation parameters. The percentage of recovery of Ciprofloxacin and Tinidazole was found to be 100.4, 101.9 at 100% level. their related substances was found to be with in acceptance range at 100% level. The standard deviation values and good recoveries indicate the reproducibility and accuracy of the developed method. As well the % RSD values for precision study also were within acceptable limit**.**

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