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Original Article

VALIDATED STABILITY INDICATING LIQUID CHROMATOGRAPHIC METHOD FOR QUANTIFICATION OF CIPROFLOXACIN HCL, ITS RELEATED SUBSTANCE AND TINIDAZOLE IN TABLET DOSAGE FORM

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ABSTRACT

Objective: A simple stability-indicating RP-HPLC method was developed for ciprofloxacin HCl, its analog ethylenediamine (EDA) and tinidazole.

Methods: In the proposed gradient method mobile phase-A (potassium dihydrogen phosphate buffer, pH 4.5) and mobile phase-B (buffer and acetonitrile in 50:50 v/v) were pumped through HPLC Waters system equipped with Empower 2 software, separation was achieved on X-terra C_{18} column (4.5 x150 mm ID, 5µm particle size) with a flow rate of 1mL/min and eluents were detected at 278 nm for ciprofloxacin HCl, it's analog EDA and 317 nm for tinidazole using PDA detector. The retention times were found to be 21.98, 18.56 and 11.02 for ciprofloxacin, EDA and tinidazole respectively. The developed method was also validated for linearity, accuracy, precision, specificity and robustness as per ICH guidelines.

Results: The linearity was found in the range of LOQ-200% and shows a correlation coefficient of 0.999 for all three substances. The LOQ for ciprofloxacin HCl, tinidazole and EDA was found to be 0.105,0.153 and 0.119 µg/mL respectively. Degradation was observed for ciprofloxacin and tinidazole during stress conditions and their peaks were well separated from the degradation product peaks, the resolution was found to be more than 2.0.

Conclusion: Hence the proposed method was a good approach for obtaining reliable results and found to be suitable for the routine analysis of ciprofloxacin HCl, tinidazole and related substance of ciprofloxacin HCl (EDA).

Keywords: Ciprofloxacin HCl, Tinidazole, Ethylenediamine analog, HPLC, Stability indicating.

INTRODUCTION

Ciprofloxacin HCl {1- Cyclopropyl-6- fluoro-1, 4-dihydro-4-oxo-7-(1-piperazinyl)-3- quinoline carboxylic acid, mono hydrochloride, monohydrate} is a synthetic fluoroquinolone, has *in-vitro* activity against a wide range of gram-negative and gram-positive microorganisms. Its bactericidal action is achieved through inhibition of topoisomerase II & IV. Upon exposure to sunlight ciprofloxacin forms ethylenediamine analog {7-[(2-aminoethyl) amino]-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid}a related substance of ciprofloxacin HCl. Tinidazole {1-[2-(ethylsulfonyl) ethyl]-2-methyl-5-nitroimidazole} is an antiprotozoal, antibacterial agent. The nitro-group of tinidazole is reduced by cell extracts of trichomonas. The free nitro-radical generated as a result of this reduction may be responsible for the antiprotozoal activity. Chemically reduced tinidazole was shown to release nitrites and cause damage to purified bacterial DNA. Ciprofloxacin HCl and tinidazole combination is approved in different regulatory authorities and marketed in several countries [1-4].

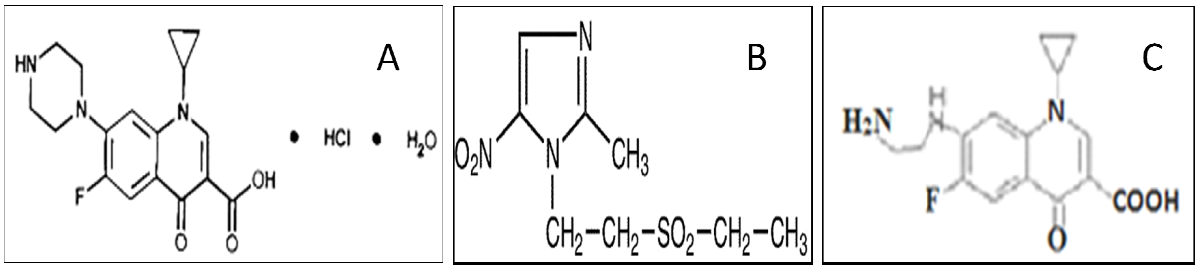


Fig. 1: Chemical structure of Ciprofloxacin HCl (A), Tinidazole (B) and Ethylenediamine analog of Ciprofloxacin(C).

Extensive literature search was carried out on analytical methods to estimate the related substances of ciprofloxacin and tinidazole. The literature survey revealed that few HPLC, UPLC and LC-MS methods had been reported for determination as well as stability studies of ciprofloxacin HCl and tinidazole combination [5-14]. Although there are few RP-HPLC methods available for ciprofloxacin HCl and tinidazole combination but none of the methods described for ciprofloxacin HCl, tinidazole and related substances of ciprofloxacin HCl (EDA analog). Hence the present work was aimed to develop and validate a related substances method for ciprofloxacin HCl and tinidazole by RP-HPLC.

MATERIALS AND METHODS

Instruments and chromatographic conditions

Analytical balance and pH meter of made Sartorius, Sonorex Sonicator and PVDF filters of made Zodiac Life Sciences were used for the study. Chromatographic separation was performed on

Waters e-module 2690, PDA detector 2998 HPLC system with empower 2 software and X-Terra RP-8 (250×4.6 mm, 5µ) column at 30°C ± 2°C was used for separation.

Reagents and Chemicals

Standard gift sample of ciprofloxacin HCl, tinidazole and related impurities were provided by DR. Reddy's Laboratories Pvt. Ltd., Hyderabad. Tablet formulation of CIPROLET-A was procured from a local pharmacy. Acetonitrile of HPLC grade, all the chemicals and reagents used were of analytical grade. High-purity water available from Millipore purification system was used.

Preparation of stock solution

Accurately weighed 25 mg of ciprofloxacin HCl and 30 mg of tinidazole were transferred to 100 mL volumetric flask. To this 50 mL of diluent was added to dissolve and mixed well. Volume made upto the mark with diluent. From this 2 mL was filtered through 0.45µm Nylon membrane filter and inject into HPLC system.

Preparation of test solution

Twenty tablets were finely powered and tablet powder equivalent to about 25 mg of ciprofloxacin (30 mg of tinidazole) was taken in a 100 mL volumetric flask. To this 50 mL of diluent was added and sonicated for 15 minutes, then diluted to volume with diluent and mixed well. From this 2 mL was filtered through 0.45µm Nylon membrane filter and inject into HPLC system.

Impurity Solution Preparation

About 5 mg of Ciprofloxacin Impurity (ethylenediamine analog compound) was accurately weighed and transferred to a 50 mL volumetric flask containing 25ml of diluent, mixed well and made up to volume with diluent. From this 5 ml of the solution was taken and made up to 20 ml with diluent.

Method Development

Based on the information available from literature survey, selected gradient programs with mobile phase- A (KH_2PO_4 buffer, pH 4.5) and mobile phase-B (Acetonitrile: KH_2PO_4 buffer, pH 4.5, 50:50v/v), different flow rate and diluents compositions were investigated in the development of HPLC method suitable for analysis of ciprofloxacin HCl, tinidazole and related substances of ciprofloxacin HCl. The suitability of the mobile phase was decided on the basis of the sensitivity of the method, suitability for stability studies, time required for the analysis, ease of preparation and use of readily available solvents. Optimization of chromatographic conditions was also done based on observations for various parameters such as retention time, theoretical plates and resolution.

Method validation

Linearity

To demonstrate the linearity for ciprofloxacin, tinidazole and EDA not less than six solutions with concentrations ranging from limit of quantification level 50% to 200% of the target concentration at specification limit were prepared. A 10 µL of each sample was injected in six times for each concentration level and calibration curve was constructed by plotting the average peak area ratio versus concentration of the standard solution.

Precision

The precision of the method was determined by analysis of six replicates of test samples prepared by spiking all the impurities at specification limit to the target concentration. These solutions were injected into the chromatographic system and individual % RSD of diluted standard and all the impurities were calculated [15].

Accuracy

Accuracy was determined by the standard addition method. Accuracy was performed in six times by spiking standard drug and EDA at 50%, 75%, 100%, 125% and 150% level and the mixtures were analyzed by the proposed method. Recovery (%) and RSD (%) were calculated for each concentration.

Specificity

Specificity to ensure that the signal measured comes from the substance of interest, and that there is no interference from excipients, degradation products and/or other impurities. Placebo solution and known impurity solution were prepared in duplicate and injected into the chromatographic system to check the interferences due to placebo, blank and other impurities at the retention time of ciprofloxacin, tinidazole and EDA analog.

Robustness

The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions included flow rate (±0.2mL/min), pH of mobile phase (±0.2) and mobile phase composition (±10%).

System suitability

The system suitability studies were carried out as specified in USP for impurity and standard solutions. The system suitability impurity solutions were prepared by using their related impurities of ciprofloxacin and tinidazole. The standard solution was prepared by using ciprofloxacin and tinidazole working standard as per test method and injected six times into the HPLC system. These parameters include column efficiency, resolution, tailing factor and number of theoretical plates were evaluated.

Forced degradation studies

A study was conducted to demonstrate the effective separation of degradents. Separate portions of drug product and placebo were exposed to induce degradation under stress conditions like acid (2N HCl), base (2N NaOH), Peroxide (5% H_2O_2), UV light (200 wts.hr/cm^2 for 55hrs), heat (105°C for 48hrs), humidity (90%H for 7 days) and water. Stressed samples were injected into the HPLC system using photo diode array (PDA) detector for above test method conditions. The chromatograms of the stressed samples were evaluated for peak purity of ciprofloxacin and tinidazole at 278nm and 317nm, respectively using proposed HPLC conditions [16-19].

RESULTS AND DISCUSSION

After investigating several chromatographic conditions for optimization of RP-HPLC method, found that potassium dihydrogen phosphate buffer, pH 4.5 as mobile phase –A and mixture of potassium dihydrogen phosphate buffer, pH 4.5 and acetonitrile in the ratio of 50:50%v/v was selected as a mobile phase-B gave retention times 21.98, 11.02 and 18.56 respectively for ciprofloxacin, tinidazole and ethylenediamine, shown in Figure 2. The column used was X-Terra C18 column (150 × 4.5mm 5µm) with flow rate 1mL/min using PDA detection at 278 nm & 317 nm. Acetonitrile: KH_2PO_4 buffer, pH 4.5 in the ratio of 70:30 (v/v) was used as diluent. These optimized chromatographic conditions for the method provides good separation of drugs and their impurity peaks by satisfying all the system suitability parameters, shown in Table 1.

Table 1: Results of system suitability parameters

Sample	Retention time	Area	USP resolution	USP Tailing	Plate count
Ciprofloxacin	21.98	14250659	-	1.46	7834
Tinidazole	11.02	6400781	10.19	0.85	9852
EDA	18.56	78345	6.15	1.02	4357

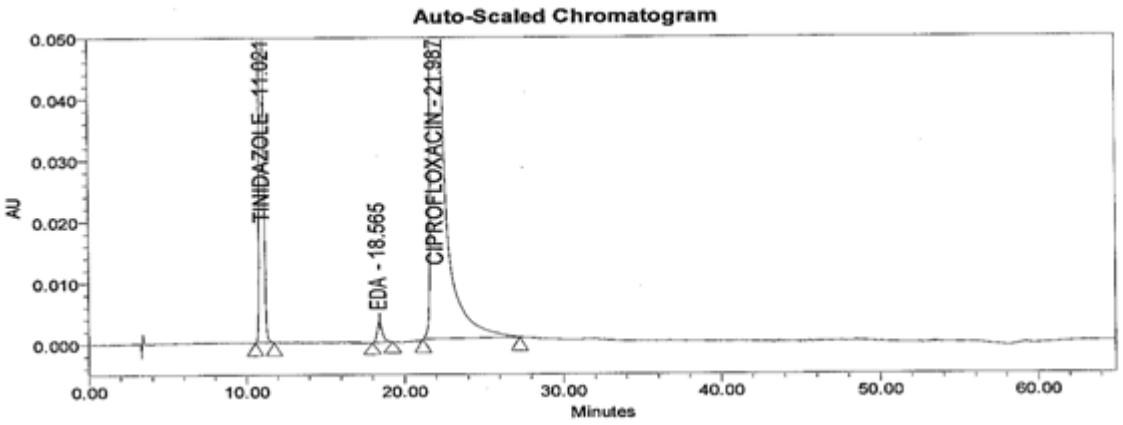


Fig. 2: Optimized chromatogram of proposed method.

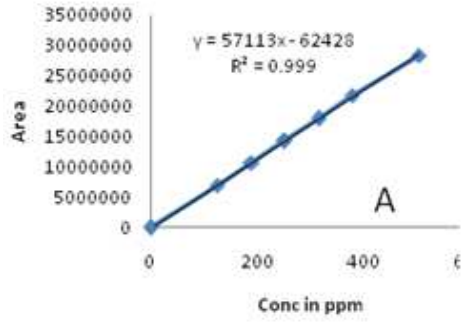
Table 2: Assay of Ciprofloxacin HCl and Tinidazole in formulation

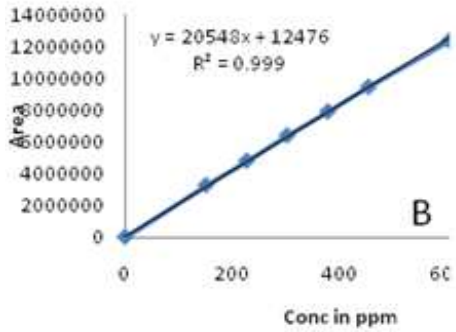
Formulation	Ciprofloxacin				Tinidazole			
	Label claim (mg)	Amount found (mg) A.M± SD	% Assay	% RSD	Label claim (mg)	Amount found(mg) A.M± SD	% Assay	% RSD
CIPROLET-A	250	252.12±0.12	100.8	0.047	300	299.5±0.458	99.83	0.152

From the results of the assay study (Table 2), the content of ciprofloxacin HCl was found to be 252.12 mg/mL (label claim percentage was 100.8 %) while the tinidazole was 299.5 mg/mL (label claim percentage was 99.98%).

Method validation

The proposed method was validated as per ICH guidelines for parameters: linearity and range, precision, accuracy, specificity, robustness, limit of detection (LOD), limit of quantitation (LOQ), system suitability and forced degradation studies.





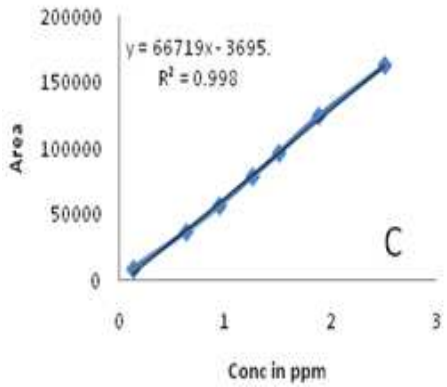


Fig. 3: Calibration plots of Ciprofloxacin HCl (A), Tinidazole (B) and Ethylenediamine analog (C)

Linearity

The linearity was evaluated by the least square regression method. The linear regression data of calibration plots for proposed method, shown in figure 3, indicated that good linear relationship between peak area versus concentration in the range of 0.125-502 µg/mL, 0.150-602 µg/mL and 0.125-2.5µg/mL for ciprofloxacin, tinidazole and EDA, respectively. The correlation coefficient was found to be 0.999.

Precision

The % RSD for ciprofloxacin, tinidazole and EDA for six preparations was found to be less than 2, indicative of precise method.

Accuracy

The mean of percentage recoveries and % RSD values were calculated and reported in Table 3. The % recoveries of ciprofloxacin, tinidazole and EDA were found to be in the range 97.4 – 99.2, 98.5-101.5 and 97.8 – 98.8, respectively which are satisfactory. The % RSD value for recovery studies all analytes was less than 2.0, which indicates the developed simultaneous method was accurate.

Table 3: Results of accuracy of the method

Recovery level (%)	Recovery of analyte	Recovery (%) AM± SD	% RSD
50	**EDA**	98.86 ± 0.46	0.46
	Ciprofloxacin	98.60 ± 0.45	0.45
	Tinidazole	99.50 ± 0.24	0.24
75	**EDA**	97.86 ± 0.85	0.86
	Ciprofloxacin	98.20 ± 0.54	0.54
	Tinidazole	100.1 ± 0.42	0.41
100	**EDA**	98.40± 1.05	1.06
	Ciprofloxacin	99.25 ± 0.68	0.68
	Tinidazole	101.5 ± 0.56	0.55
125	**EDA**	98.03 ± 0.81	0.82
	Ciprofloxacin	98.40 ± 0.25	0.25
	Tinidazole	98.50± 0.84	0.85
150	**EDA**	98.20± 0.20	0.20
	Ciprofloxacin	97.43 ± 0.23	0.24
	Tinidazole	100.1 ± 0.17	0.16

Table 4: Results of robustness study of the method

Factor	**Retention Time**			**Theoretical plates**		
Analyte	**EDA**	**CIP**	**TIN**	**EDA**	**CIP**	**TIN**
Flow rate (mL/min)						
0.8	18.0	21.2	11.5	4231	7896	9629
1.0	18.4	21.9	11.0	4357	7834	9852
1.2	18.2	20.5	11.2	4198	7781	9743
% Of Acetonitrile						
45	18.1	21.6	11.2	4306	7754	9718
50	18.4	21.9	11.0	4357	7834	9852
55	18.5	20.8	10.7	4285	7881	9907
pH of the mobile phase (pH 4.5)						
pH 4.3	18.0	21.2	10.8	4190	7649	9682
pH 4.5	18.4	21.9	11.0	4357	7834	9852
pH 4.7	18.6	21.5	11.1	4408	7716	9889

Table 5: Results of forced degradation studies

Stress Condition	**Ciprofloxacin**				**Tinidazole**			
	%Net Degradation	**Purity Angle**	**Purity Threshold**	**Purity Flag**	**% Net Degradation**	**Purity Angle**	**Purity Threshold**	**Purity Flag**
Acid degradation	1.9	0.111	0.269	No	0.7	0.055	0.243	No
Base degradation	3.3	0.123	0.269	No	1.6	0.050	0.242	No
Peroxide degradation	2.7	0.082	0.264	No	0.0	0.040	0.242	No
Heat degradation	0.9	0.101	0.261	No	0.1	0.044	0.239	No
Sunlight degradation	0.9	0.092	0.266	No	0.5	0.042	0.243	No
Water degradation	2.0	0.100	0.264	No	2.8	0.041	0.241	No
Humidity degradation	2.6	0.098	0.262	No	3.5	0.043	0.239	No
UV light	1.1	0.095	0.261	No	0.7	0.042	0.240	No

Limit of detection (LOD) & limit of quantification (LOQ)

Limit of detection (LOD) and Limit of Quantification (LOQ) were determined by S/N ratio method. LOD was found to be 0.010 µg/mL, 0.015µg/mL and 0.062 µg/mL for ciprofloxacin, tinidazole and EDA, respectively (S/N ratio 3:1).LOQ was found to be 0.105 µg/mL, 0.150 µg/mL and 0.125 µg/mL for ciprofloxacin, tinidazole and EDA, respectively (S/N ratio 10:1). The mean recovery of ciprofloxacin, tinidazole and ethylenediamine analog, at limit of Quantification level was 94.8%, 100.4% and 101.9% respectively.

Robustness

Robustness of the method was performed by slightly varying the chromatographic conditions. The results showed insignificant effect on the chromatographic parameters by slight variations in chromatographic conditions with respect to mobile phase, flow rate and pH are presented in Table 4.

Forced degradation studies

Degradation was observed for ciprofloxacin and tinidazole during stress conditions and their peaks were well separated from the degradation product peaks, the resolution was found to be more than 2.0. The degradation data was shown in Table 5 revealed that ciprofloxacin degradation was more in basic than other stress conditions and tinidazole degradation was very less in all stress conditions. In all forced degradation samples, the purity angle found less than purity threshold. This indicates that there is no

interference from degradents at ciprofloxacin and tinidazole peaks. Thus, this method is considered to be "Stability Indicating Method".

CONCLUSION

A simple precise and accurate method was developed for the estimation of EDA analog of ciprofloxacin, ciprofloxacin HCl and tinidazole. The developed method utilizes less organic solvent and it has high resolution, more sensitive with reduced run time. This RP-HPLC method is also validated for various parameters as per ICH guidelines. The accuracy was found to be within the limits. The linearity regression data showed a good linear relationship over a range of LOQ-200 % for ciprofloxacin, tinidazole and related substance of ciprofloxacin HCl (EDA analog). The method is robust as observed from insignificant variation in the results of analysis on changes in flow rate, mobile phase composition and pH of the buffer. The system suitability parameters proved that the proposed method is suitable for estimation of ciprofloxacin HCl, tinidazole and related substance of ciprofloxacin HCl (EDA analog). Forced degradation Studies were done for ciprofloxacin HCl and tinidazole under stress conditions to demonstrate that the method is stability indicating. Good agreement was seen in the estimation of results in combined dosage form for its related substance by developed method. Hence it is concluded that the developed RP-HPLC method can be effectively used for estimation of ciprofloxacin HCl, tinidazole as well as the ciprofloxacin ethylenediamine analog from pharmaceutical dosage forms.

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