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ASSESSMENT OF CIPROFLOXACIN CONTENT VARIATION IN ANTIMICROBIAL SUSCEPTIBILITY TEST DISCS BY LIQUID CHROMATOGRAPHY

Mahathi Kothapalli*[1], Gurubasavaraja swamy PM[1], V.N.Raju Erumalla[2], Rajesh Telugu[3], Swetha Chenna[4]

[1]Department of Quality Assurance, Acharya & B.M.Reddy College of Pharmacy Soladevanahalli, Hesaraghatta, Bangalore-560107, India.
[2]Department of Biotechnology & Microbiology,Acharya & B.M.Reddy College of Pharmacy Soladevanahalli, Hesaraghatta, Bangalore-560107, India.
[3]Department of Industrial Pharmacy,Acharya & B.M.Reddy College of Pharmacy Soladevanahalli, Hesaraghatta, Bangalore-560107, India.
[4]Institute of Bioinformatics and Applied Biotechnology, Electronics city, Bangalore -560100, India.

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***Correspondence for Author**
Mahathi Kothapalli
Department of Quality Assurance, Acharya & B.M. Reddy College of Pharmacy Soladevanahalli, Hesaraghatta, Bangalore-560107, India.

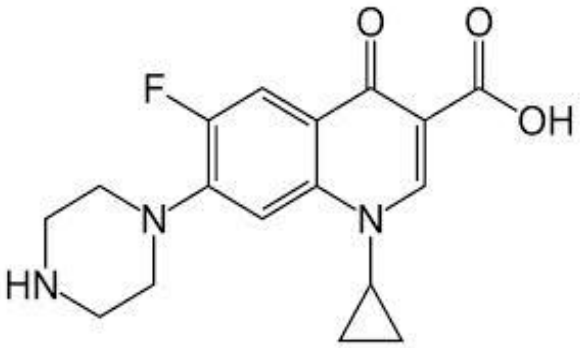
ABSTRACT

A new simple, rapid, selective, precise and accurate isocratic reverse phase high performance liquid chromatography assay has been developed for the estimation of Ciprofloxacin bulk powderand in discs. The separation was achieved by using C-18 column (Phenomenex Luna C_{18} column 250 mm×4.6mm id, 5µ particle size) coupled with a guard column of silica in mobile phase ammonium acetate buffer and acetonitrile (25mM ammonium acetate buffer with 75:25). The flow rate was 1.0ml/min and the drug was detected using UV detector at the wavelength of 275nm. The retention time was within 2.468 - 2.473 minutes. The method was validated as per ICH guidelines. Antimicrobial activity viz., Minimum inhibitory concentration (MIC) and the Minimum bactericidal concentration (MBC) against *S. aureus and E. coli* was subjected by using ciprofloxacin bulk powder and discs, and the results were interpreted by determining the zone of inhibition using Disc diffusion method of two different batches of 10µg and 30µg of ciprofloxacin discs from the same manufacturer.The Propose HPLC method is simple, precise, accurate, robust, significant and suitable for determining the assessment of ciprofloxacin content variation in antimicrobial susceptibility test discs by liquid chromatography.

KEY WORDS: High Performance Liquid Chromatography, Ciprofloxacin, Zone of inhibition, Minimum inhibitory concentration, *S. aureus, E. coli.*

INTRODUCTION

Worldwide medical microbiological laboratories have adopted the British Society for Antimicrobial Chemotherapy (BSAC) method for antimicrobial disc susceptibility testing[1] in common with a number of other testing methods. [1-3]When the combination discs, containing antibiotic mixtures (Cefpodoxime /clavulanic acid combination disc 10/1µg) are tested, organisms are to be employed in such a way that synergistic action is not observed.[4]As per the guidelines of the WHO Expert Committee on Biological Standardizationthe manufacturing standards for antimicrobial susceptibility testing discs permit a range of content from 90% to 120% (± 30%) of the stated disc content.[5] This allows manufacturers to load discs above the stated content to allow for the loss on storage and prolong shelf lives. The variability in disc content affects the zone diameter greatly and thus the results obtained therein. Ciprofloxacin (CPX) is a fluorinated quinolone antibacterial which is chemically 1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-quinoline-3-carboxy-lic acid (The Merck Index, 2006). Ciprofloxacin is a broad spectrum antibiotic active against both Gram-positive and Gram-negative bacteria. It functions by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV enzymes necessary to separate bacterial DNA, thereby inhibiting cell division. [6]



Ciprofloxacin

Literature survey revealed that various analytical methods such as UV spectroscopy; [7-8] HPLC [9], pulsepolarography [10] have been reported for the estimation of the drugs. HPLC methods offer improved accuracy and greater precision when compared to the standard methods for analysis of antibiotics present alone or in combination in these discs. The aim of the present investigation is to develop a liquid chromatographic method for assessing the content variability of ciprofloxacin in antimicrobial susceptibility test discs and hence an economical method was developed and validated according to the ICH guidelines.

MATERIALS AND METHODS

Instruments

Absorbance measurements were made on RP-HPLC (SPD 20AUV-visible detector and LC-20AT) instrument, Digital analytical balance (AUX 220, Shimadzu) for weighing and Ultra sonicator (Dikshin, Mumbai)were used.

Chemicals and reagents

All chemicals were of analytical reagent grade and solutions were prepared with double distilled water. Ciprofloxacin gift samples was obtained from Dr. Reddy's Laboratories, Hyderabad. HPLC grade ammonium acetate buffer, HPLC grade water, HPLC grade methanol, HPLC grade acetonitrilewere procured from Finar chemicals Ltd., Ahmedabad and Merk specialities private Ltd., Mumbai, India. Beef extract, Peptone, Sodium chloride and Dimethyl sulphoxide were procured from Central Drug House Pvt Ltd, New Delhi andQualigens fine chemicals Ltd, Mumbai. *S. aerous and E.coli* were procured from IMTECH Chandigarh.Ciprofloxacindiscs were procured from the market retail outlet manufactured by Himedia Laboratories Pvt Ltd Mumbai.

METHODS

Preparation of 25 mM ammonium acetate bufferand Acetonitrile stock solution

Accurately weigh about 192.5mg ofAmmonium acetate, dissolve in distilled water and make up the volume to 100ml of HPLC grade water. Add 100ml of acetonitrile to 100ml of HPLC grade water,both of the above solutions were filtered and sonicated for 10 minutes prior to use.

Preparation of stock solution (1000µg/ml)

Accurately weighed quantity of pure Ciprofloxacin (10mg) and was transferred intoseparate10ml volumetric flasks, dissolved in methanol and made up the volume to 10ml with the same solvent. The stock solution was sonicated for 2minutes.

Preparation of stock Disc solution

10 discs of two batches of 10µg and 30µg average weight was taken and weight of single disc was taken and one disc in 1ml of methanol was added and filtered and sonicated for 10 minutes prior to use. Then the extract was injected at 20µl 0f 10µg/ml. [11-15]

Conditions of Chromatographic Method

Chromatographic separation was performed using an isocratic elution at 40°C on C-18 column parked with silica. The mobile phase was composed of 25mM ammonium acetate buffer with and acetonitrile (75:25v/v) and pH of mobile phase was adjusted to 5.0 ± 0.1 with acetic acid. The flow rate is 1.0ml/min and the injection volumewas 20µl. UV measurement was made at wavelength of 275nm.

Antimicrobial susceptibility test

Antimicrobial susceptibility was tested against *S.aureus* and *E.coli*by using LB media. The Minimum inhibitory concentration (MIC) and the Minimum bactericidal concentration (MBC) values were determined by serial micro dilution assay. All the media prepared was then sterilized by autoclaving the media at 121°C, 15lbs for 15 minutes.The screening of the ciprofloxacin powder for antibacterial effect was carried out by determining the zone of inhibition using Disc diffusion method of two different batches of 10µg and 30µg of ciprofloxacin discs. Sterile nutrient agar plates were prepared. Then 0.1ml of test organism *(S.aureus and E. coli)* was taken from the stock (broth) and swabbed on the agar medium in aseptic condition. The filter paper disc of 2mm diameter (Whatman No.1 Filter paper) were prepared and sterilized. The Ciprofloxacin bulk powder to be tested were prepared with concentration at 10µg/ml was added to each disc of holding capacity of 10µl. The sterile impregnated disc with Ciprofloxacin powder were placed on the agar surface with framed forceps and gently pressed down to ensure complete contact of the Ciprofloxacin disc with the agar surface. Positive control disc of Ciprofloxacin was prepared and placed on the agar surface. All the plates were incubated at 37°C for 24 hours. After incubation, the size (diameter) of the inhibition zones was measured and calculated[16-20].

RESULTS AND DISCUSSION

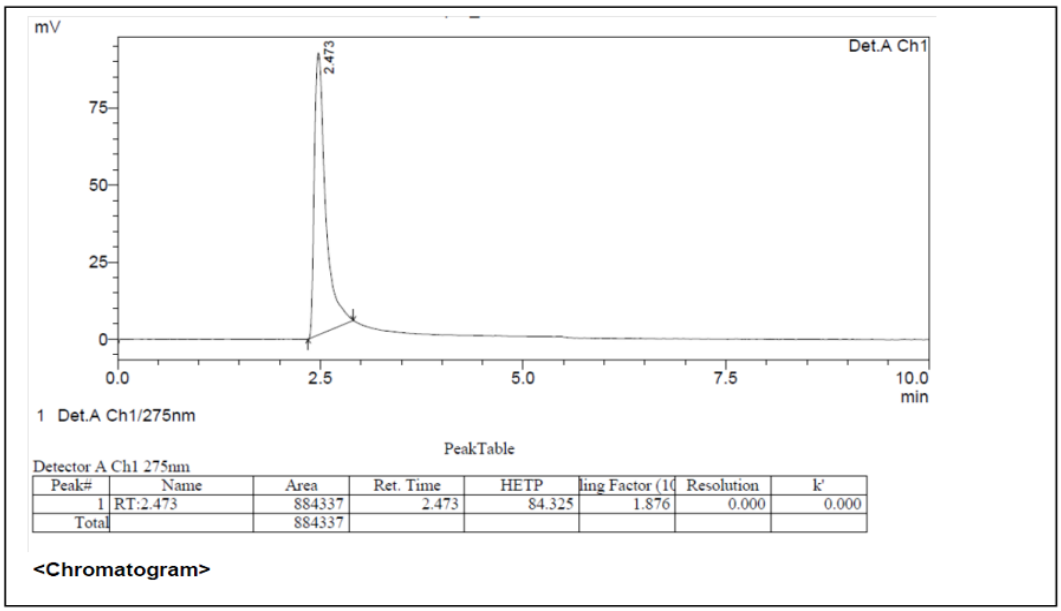
The main objective of the present work to develop a new Liquid Chromatographic (LC) method for the estimation of ciprofloxacin discs. To employ the LC method developed for estimation of ciprofloxacin content in antimicrobial susceptibility test discs of 10µg and 30µg concentrations. To assess the Inter-Batch variation in the content of ciprofloxacin in antimicrobial susceptibility test bulk powder of 10µg and discs (Fig:1) of 2 different batches of 10µg & 30µg concentrations purchased from the same manufacturer (Fig: 2-5).With these objectives the HPLC method was developed successfully and then validated based on ICH parameters[21-23]. The parameters was studied to validate the developed HPLC method were

linearity, precision, specificity, accuracy, limit of detection, limit of quantitation and robustness.Shimadzu LC 20 AT system equipped with Phenomenex Luna C_{18} column (250mm×4.6mm ID, 5µm particle size)[24-28], and SPD 20A UV/Vis detector was used for the study. The mobile phase used was 25mM ammonium acetate buffer and acetonitrile with the ratio of (75:25v/v), at the flow rate 1.0 ml/min and UV detection at 275nm.

Linearityof the drug was found to be in the concentration range of 0.5-5.0µg/ml for Ciprofloxacin. Regression coefficient (r^2) was within the limits and found to be 0.9984 (Fig:6).Precisionof the method was determined by replicate injection of sample solutions. The Intra-day % RSD and Inter-day % RSD was found to be as 0.1-0.4and 0.2-0.4 respectively for Ciprofloxacin, which was within the acceptance criteria % RSD $\leq$ 2. Specificity of the method was determined. Chromatogram of placebo showed no peaks at the retention time of Ciprofloxacin 2.473. This indicates that the excipients used in the formulation do not interfere in the estimation of Ciprofloxacin. Accuracy was determined through recovery studies of Ciprofloxacin. The recovery of Ciprofloxacin discs was found to be 99-100.6%, which was found to be well within the acceptance limit of 95-105%.

Robustness of the method was determined by deliberately changing mobile phase ratio, mobile phase and wavelength were altered slightly in HPLC method.System suitability was determined by the number of theoretical plates per 2830.490. The symmetry or the tailing factor was found to be 1.876. To ensure the variation in the content of Ciprofloxacin discs the microbiological Assay was carried out and the results were compared.It is an alternative procedure and the one which eventually was adopted in laboratory is a single disc assay procedure which calls for the extraction of the content of one disc into 1ml of methanol and the samples are analyzed and the data are averaged to yield the appropriate potency value.The Antimicrobial studies were done and it is tested on solid Agar-agar media. The Minimum inhibitory concentration (MIC) and the Minimum bactericidal concentration (MBC) values were determined by serial micro dilution assay[29-30]. All the media prepared was then sterilized by autoclaving the media at 121°C for 20 minutes. The screening of the ciprofloxacin bulk powder for antibacterial effect was carried out by determining the zone of inhibition using Disc diffusion method of two different batches of 10µg and 30µg of ciprofloxacin discs. Sterile nutrient agar plates were prepared. Then 0.1ml of test organism (*S.aureus and E. coli*) was taken from the stock (broth) and swabbed on the agar medium in aseptic condition.Positive control of Ciprofloxacin was prepared and placed on the agar

surface. All the plates were incubated at 37°C for 24 hours. After incubation, the size (diameter) of the inhibition zones was measured and calculated (Table: 1-4 and Fig: 7-10).



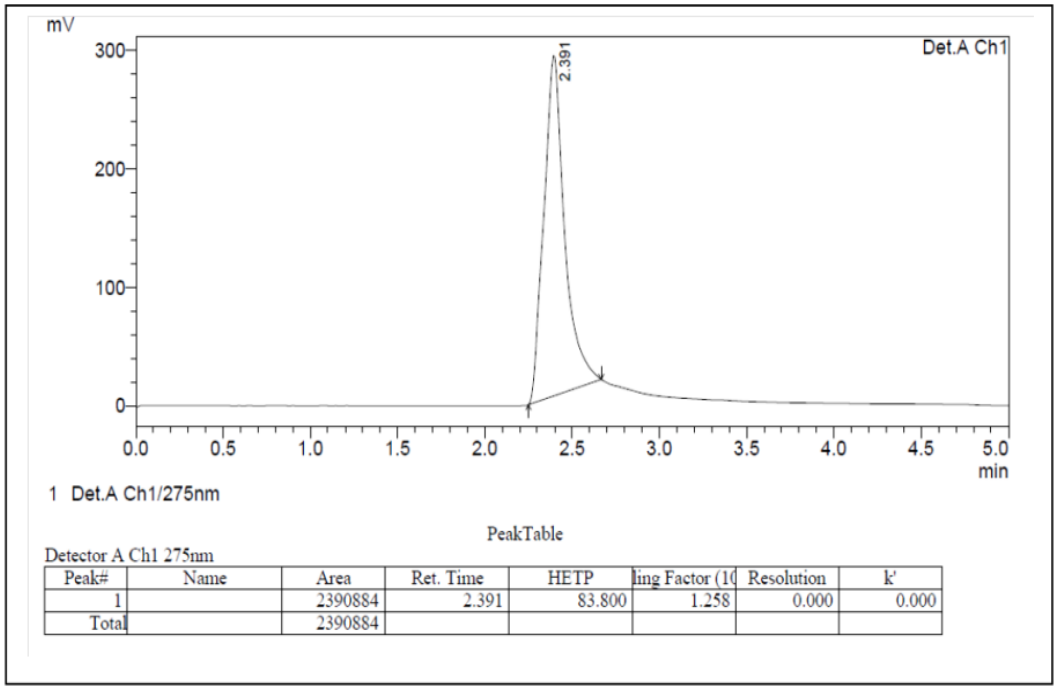
PeakTable

Detector A Ch1 275nm

Peak#	Name	Area	Ret. Time	HETP	ling Factor (1(	Resolution	k'
1	RT:2.473	884337	2.473	84.325	1.876	0.000	0.000
Total		884337					

<Chromatogram>

Fig: 1. Chromatogram of Ciprofloxain Standard drug (10µg/ml)

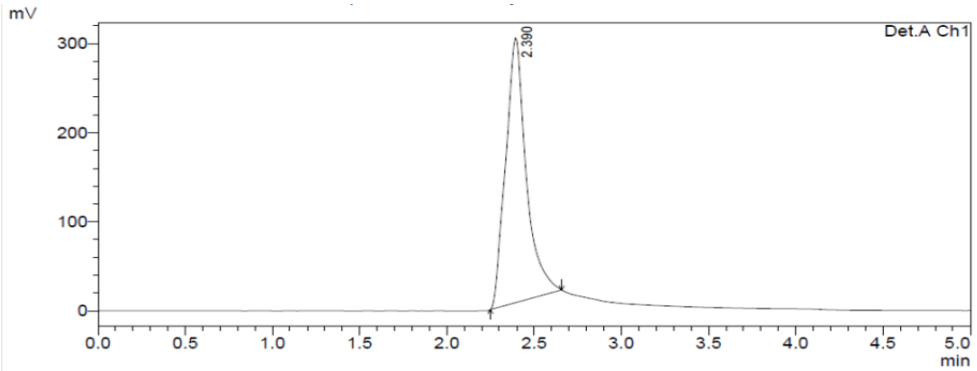


PeakTable

Detector A Ch1 275nm

Peak#	Name	Area	Ret. Time	HETP	ling Factor (1(	Resolution	k'
1		2390884	2.391	83.800	1.258	0.000	0.000
Total		2390884					

Fig: 2.Chromatogram of Ciprofloxacin discs 10µg - (170390)

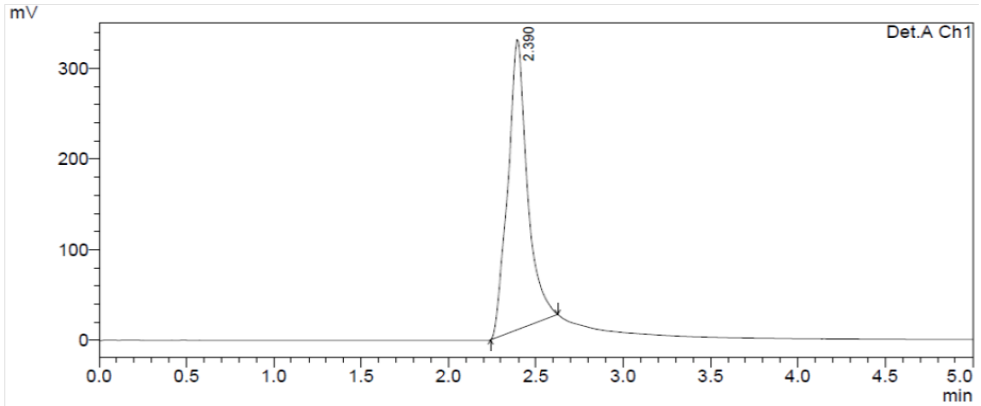


PeakTable

Detector A Ch1 275nm

Peak#	Name	Area	Ret. Time	HETP	ling Factor (1(	Resolution	k'
1		2454029	2.390	83.515	1.236	0.000	0.000
Total		2454029					

Fig: 3.Chromatogram of Ciprofloxacin discs 10µg - (187107)

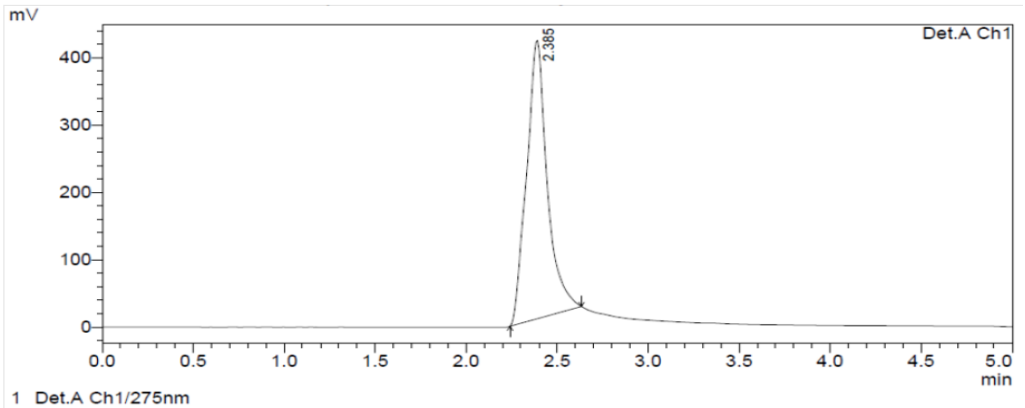


PeakTable

Detector A Ch1 275nm

Peak#	Name	Area	Ret. Time	HETP	ling Factor (1(	Resolution	k'
1		2502165	2.390	72.709	1.162	0.000	0.000
Total		2502165					

Fig:4. ChromatogramofCiprofloxacindiscs30µg-(171343)



PeakTable

Detector A Ch1 275nm

Peak#	Name	Area	Ret. Time	HETP	ling Factor (1(	Resolution	k'
1		3270275	2.385	77.300	1.163	0.000	0.000
Total		3270275					

Fig: 5.Chromatogram of Ciprofloxacin discs 30µg - (156853)

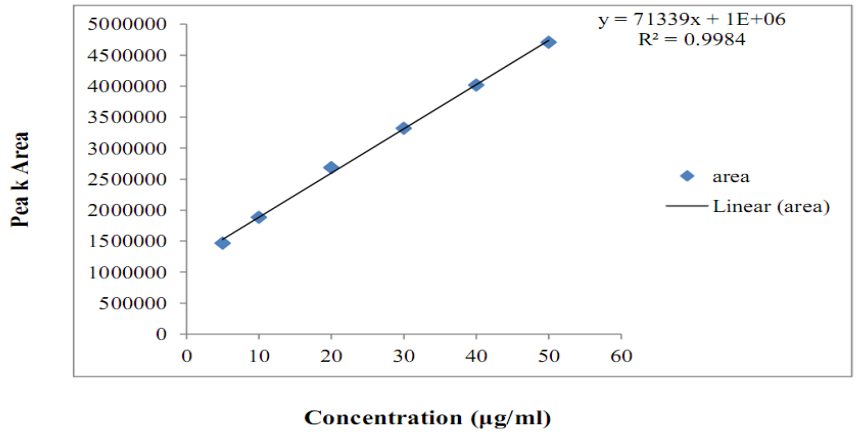


Fig: 6. Linearity graph for Ciprofloxacin standard drug

Table: 1. MBC studies Ciprofloxacin disc of *E. coli* (-ve)

Concentration(µg)	Standard	Test Organism (*E. coli*)	MBC
10	1.1cm	1.3cm	8.46
10	1.2cm	1.3cm	9.23
30	1.3cm	1.5cm	22.57
30	1.2cm	1.6cm	22.52

Table: 2. MBC studies Ciprofloxacin disc of *S.aureus* (+ve)

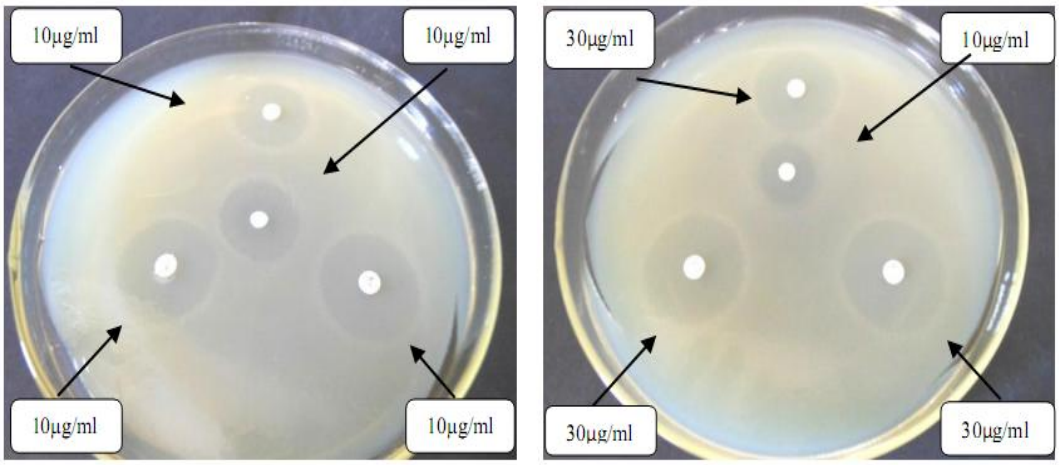
Concentration(µg)	Standard	Test Organism (*S.aureus*)	MBC
10	1.1cm	1.4cm	9.28
10	1.2cm	1.3cm	9.23
30	1.3cm	1.6cm	22.58
30	1.2cm	1.6cm	22.49

Table: 3. MIC studies Ciprofloxacin disc of *S.aureus* (+ve)

Concentration(µg)	Standard	Test Organism (*S.aureus*)	MIC
10	1.2cm	1.3cm	8.79
10	1.3cm	1.6cm	7.96
30	1.1cm	1.4cm	24.77
30	1.3cm	1.6cm	23.89

Table: 4. MIC studies Ciprofloxacin disc of *E.coli* (-ve)

Concentration(µg)	Standard	Test Organism (*E.coli*)	MIC
10	1.3cm	1.3cm	8.42
10	1.4cm	1.6cm	8.49
30	1.3cm	1.6cm	23.41
30	1.2cm	1.7cm	23.89



Test = Ciprofloxacin disc (10µg/ml)

STD = Ciprofloxacin disc (10µg/ml)

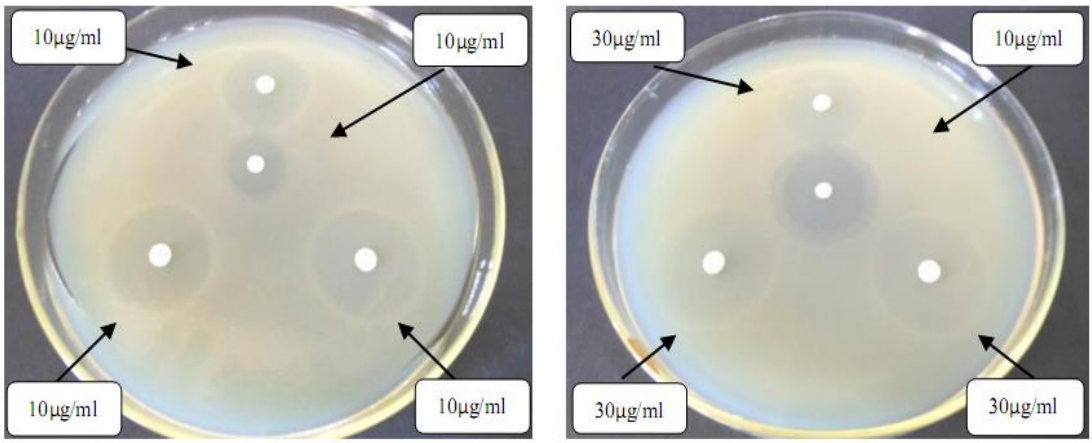
Organism = *E. coli*

Test = Ciprofloxacin disc (30µg/ml)

STD = Ciprofloxacin disc (10µg/ml)

Organism = *E. coli*

Fig: 7.MBC studies Ciprofloxacin disc of *E. coli* (-ve)



Test = Ciprofloxacin disc (10µg/ml)

STD = Ciprofloxacin disc (10µg/ml)

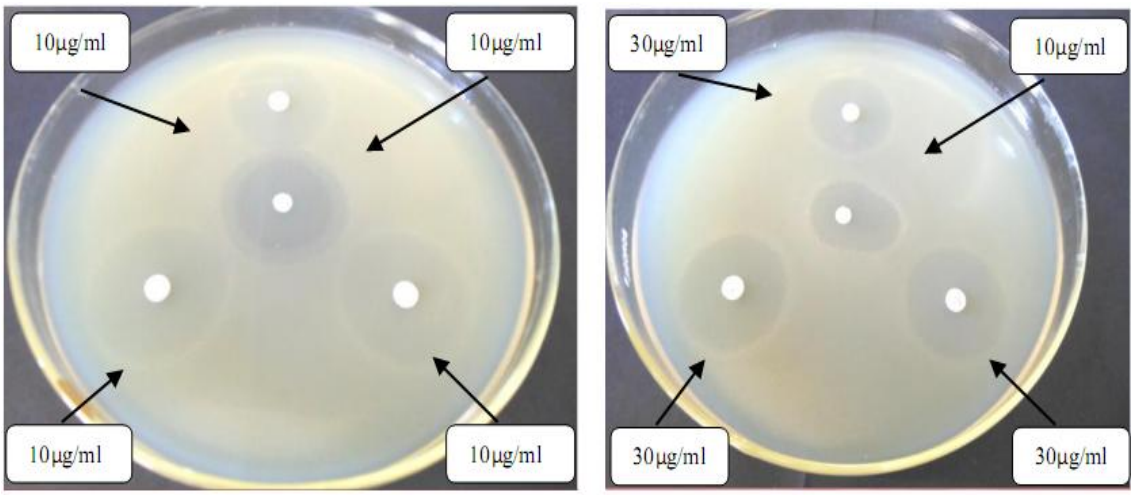
Organism = *S. aerous*

Test = Ciprofloxacin disc (30µg/ml)

STD = Ciprofloxacin disc (10µg/ml)

Organism = *S. aerous*

Fig: 8. MBC studies Ciprofloxacin disc of *S.aureus* (+ve)

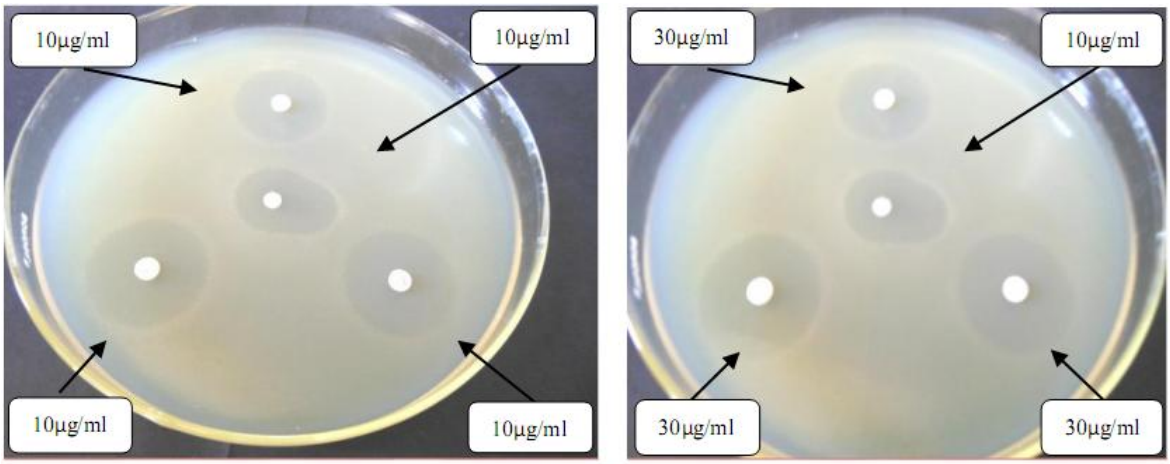


Test = Ciprofloxacin disc (10µg/ml) Test = Ciprofloxacin disc (30µg/ml)

STD = Ciprofloxacin disc (10µg/ml) STD = Ciprofloxacin disc (10µg/ml)

Organism = *E. coli* Organism = *E. coli*

Fig: 9. MIC studies Ciprofloxacin disc of *E. coli* (-ve)



Test = Ciprofloxacin disc (10µg/ml) Test = Ciprofloxacin disc (30µg/ml)

STD = Ciprofloxacin disc (10µg/ml) STD = Ciprofloxacin disc (10µg/ml)

Organism = *S. aerous* Organism = *S. aerous*

Fig: 10. MIC studies Ciprofloxacin disc of *S. aureus* (+ve)

CONCLUSION

The Quantitation of Ciprofloxacin was achieved by measuring the peak area and the 2 different batches of 10µg & 30µg concentrations of Ciprofloxacin discs were taken for the analysis and the assay is performed individually and results were achieved. The specificity study indicates that the excipients present in the formulation do not interfere in the analysis. To ensure the variation in the content of Ciprofloxacin discs the Microbiological Assay was carried out and the results were compared.It is an alternative procedure and the one which eventually was adopted in laboratory is a single disc assay procedure which calls for the extraction of the content of one disc into 1ml of methanol and the samples are analyzed and the data are averaged to yield the appropriate potency value.The Proposed HPLC method is simple, precise, accurate, robust, significant and suitable for determining the assessment of ciprofloxacin content variation in antimicrobial susceptibility test discs by liquid chromatography.Buffer was used to save life of the column and the single disc assay is more time consuming.It is helpful for laboratories when assessing which of the two discs contents permitted in the method should be used and the assay can be applied to the pharmacokinetic study of the patients with Ciprofloxacin treatment.

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