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Design of Experiment Utilization to Develop a Simple and Robust RP-UPLC Technique for Stability Indicating Method of Ciprofloxacin Hydrochloride and Metronidazole in Tablets

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Abstract

Ciprofloxacin Hydrochloride and Metronidazole are indicated for the treatment of intra-abdominal and pelvic infections caused by E. coli and diverticulitis. It is necessary to establish a Validated HPLC method for the assay of them. Using of DOE techniques provides more information about different factors affects system suitability of new method. DOE led to highly robust methods through creating design space. The method was performed on Phenomenex Kinetex C18 (50x4.6 mm, 2.6 µm) and the mobile phase consisted of triethylamine (TEA), (0.5 % v/v, pH 4.5) and acetonitrile which pumped at a flow rate 0.6 $mLmin^{-1}$ at 50 °C in gradient manner. 2 µL of drugs sample solutions were monitored at fixed wavelengths 320 nm. Ciprofloxacin Hydrochloride, Metronidazole and Caduet tablets bulk powders were stressed under different conditions in forced degradation studies. Major degradation products were identified such as 2-methyl-4-nitroimidazole, Fluoroquinolonic acid and Ciprofloxacin Ethylenediamine. The proposed method was validated in terms of linearity, accuracy, precision and limits of detection and quantitation according to ICH.

Keywords:

Ciprofloxacin Hydrochloride; Metronidazole; UPLC; Stability Indicating Method; Design of Experiment

1. Introduction

Ciprofloxacin Hydrochloride is chemically described as (1-Cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1, 4-dihydroquinoline-3-carboxylic acid hydrochloride as shown in Fig 1. It is active against both Gram-positive and Gram-negative bacteria [1].

Metronidazole is chemically described as 2-(2-Methyl-5-nitro-1H-imidazol-1-yl) ethanol as shown in Fig 1. It is an antimicrobial drug with high activity against anaerobic bacteria and protozoa [1].

USP 2014 and BP 2014 described RP- HPLC methods for estimation of both drugs individually [2, 3].

Ciprodiazole® Tablets contain 500 mg Ciprofloxacin Hydrochloride and 500 mg Metronidazole per tablet. It is indicated for the treatment of diverticulitis, intra-abdominal and

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pelvic infections caused by E. coli, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumonia or Bacteroides fragilis [4].

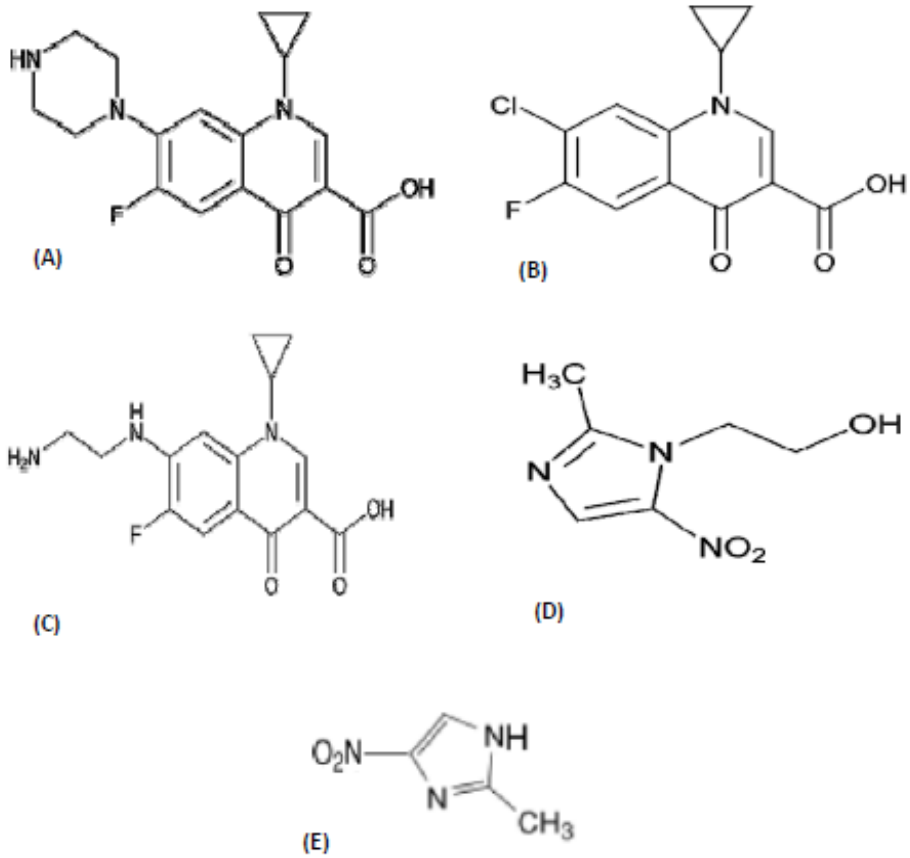


Fig.1. Structures of (A) Ciprofloxacin Hydrochloride, (B) Fluoroquinolonic acid and (C) Ciprofloxacin Ethylenediamine, (D) Metronidazole, (E) 2-methyl-5-nitroimidazole respectively. Ciprofloxacin impurity A (Fluoroquinolonic acid) (BP) is 7-chloro-1-cyclopropyl-6-fluoro-4-oxo-1, 4- dihydroquinoline-3-carboxylic acidc; Ciprofloxacin impurity C (Ethylenediamine compound) (BP) is 7-[(2-aminoethyl) amino]-1- cyclopropyl-6-fluoro-4-oxo-1, 4-dihydroquinoline-3- carboxylic acid; Metronidazole impurity A(BP) is 2-methyl-5-nitroimidazole

Literature review revealed that some methods have been reported for determination of Ciprofloxacin and Metronidazole in tablets simultaneously by Spectrophotometry [5-7], HPLC [8, 9] and calorimetric technique [10]. Literature review reveals that there is no stability indicating method for this combination. The purpose of this study was to develop a robust method as a stability indicating assay by UPLC.

Design of experiments (DOE) technique will be utilized to achieve this purpose because it has more advantages than one-factor-at-a-time (OFAT) technique. DOE enables many factors to be studied simultaneously and it achieves structured analysis of main effect, interactions and noise.

It is efficient in terms of the amount of information obtained given the number of run made and it has rigorous statistical methods for the analysis. DOE technique was employed to study the effect of critical factors on the method performance.

2. Experimental

2.1. Instrumentation

Analysis was performed on a chromatographic system of Waters Acquity UPLC equipped with a binary solvent delivery pump, an auto sampler and connected to TUV detector. The system equipped by Empower PC program (Waters, Milford, USA). The chromatographic separation was achieved on a Phenomenex Kinetex C18 (50x4.6 mm, 2.6 µm) column.

Method development and method modeling was performed using MODDE 9 Trial version 2014 software

2.2. Chemicals and reagents

All reagents used were of analytical grade or HPLC grade. 1- Hexane sulphonic acid sodium, triethylamine (TEA), perchloric acid, potassium dihydrogen phosphate and ortho phosphoric acid were supplied by Merck (Darmstadt, Germany). Acetonitrile HPLC grade was supplied by Fischer scientific (U.K.) and Distilled water was obtained from Milli-RO and Milli-Q systems (Millipore, Bedford, MA). Ciprofloxacin HCl and Metronidazole working standard powders were kindly supplied by Egyptian international pharmaceutical industries company (EIPICO) (10th of Ramadan, Egypt), and were used without further purification.

2.3. Pharmaceutical preparation

Ciprodiazole® Tablets (Minapharm, Egypt) contain 500 mg Ciprofloxacin Hydrochloride and 500 mg Metronidazole per tablet, B.NO: EFE2084.

2.4. Chromatographic condition

2 µL of drugs sample solutions were monitored at a fixed wavelength 320.0 nm. Liquid chromatography was performed on a Phenomenex Kinetex C18 (50x4.6 mm, 2.6 µm) column and the mobile phase was consisted of triethylamine (TEA), (0.5 % v/v, pH 4.5) and acetonitrile which pumped at a flow rate 0.6 mLmin^{-1} at 50 °C in a gradient manner as shown in Table1.

Triethylamine (TEA) (0.5%v/v) was prepared by dissolving 5 ml in approximately 1000 mL distilled water. The pH was adjusted to 4.5 with ortho phosphoric acid. The mobile phase was filtered through a 0.45 µm Nylon membrane filter (Millipore, Milford, MA, USA) under vacuum and degassed by ultrasonication (Cole Palmer, Vernon Hills, USA) before usage.

Table 1: Time Table of Validated Gradient Method

Time minutes	Flow Rate mL/ minute	Acetonitrile %	Buffer %
0	0.6	5	95
4	0.6	30	70
6	0.6	50	50
7	0.6	50	50
7.5	0.6	5	95
9	0.6	5	95

2.5. Preparation of stock standard solutions

Stock standard solutions contain 500 µg mL^{-1} of Ciprofloxacin Hydrochloride and Metronidazole was prepared by dissolving 50 mg of each in distilled water in 100 mL

volumetric flask respectively. It was then sonicated for 10 minutes and the final volume of solutions was made up to 100 mL with distilled water to get stock standard solutions.

2.6. Preparation of calibration plot (working standard solutions)

To construct calibration plots, the stock standard solutions were diluted with distilled water to prepare working standard solutions in the concentration ranges (1.25 -75 μg mL^{-1} of Ciprofloxacin Hydrochloride and Metronidazole. Each solution (n=10) was injected in triplicate and chromatographed under the mentioned conditions above. Linear relationships were obtained when average drug standard peak area were plotted against the corresponding concentrations for each drug. Regression equation was computed.

2.7. Sample preparation

Twenty Ciprodiazole® tablets were prepared by grinding it to a fine and uniform size powder. They were triturated using mortar and pestle. After calculating the average tablet weight, amounts of powder equivalent to 50 mg of Ciprofloxacin Hydrochloride and Metronidazole was accurately weighed and transferred separately to 100 mL volumetric flasks contain 0.05 mol L^{-1} edetate disodium. The solutions were sonicated for 15 min and the solutions were then filtered through 0.45 μm Nylon membrane filters (Millipore, Milford, MA, USA). Aliquots of appropriate volume (10 mL) were transferred to 100 mL volumetric flasks and diluted to volume with 0.05 mol L^{-1} edetate disodium to obtain the mentioned concentration above. The diluted solutions were analyzed under optimized chromatographic conditions.

2.8. Forced degradation of Ciprofloxacin Hydrochloride and Metronidazole

To determine the proposed method as a stability-indicating method for Ciprofloxacin Hydrochloride and Metronidazole and Ciprodiazole® tablets bulk powders, all of them were stressed under different conditions in forced degradation studies. Stock solutions of Ciprofloxacin Hydrochloride, Metronidazole and Ciprodiazole® tablets bulk were prepared by dissolving them in water for Ciprofloxacin Hydrochloride, Metronidazole or in 0.05 mol L^{-1} edetate disodium for Ciprodiazole® tablets respectively [11, 12].

2.8.1. Acidic degradation

Hydrochloric acid (HCl) (1 mol L^{-1}, 10 mL) was added to 10 mL prepared stock solutions of Ciprofloxacin Hydrochloride, Metronidazole or Ciprodiazole® tablets respectively. These solutions were separately heated at 75C° for 4 hours in the dark (to exclude the possible degradative effect of light). The solutions (2 mL) were then transferred to 10 mL volumetric flasks, neutralized by addition of 1mL of 1 mol L^{-1} NaOH and diluted to final volume with distilled water or 0.05 mol L^{-1} edetate disodium respectively [11, 12].

2.8.2. Alkaline degradation

Sodium hydroxide (NaOH) (1 mol L^{-1}, 10 mL) was added to 10 mL prepared stock solutions of Ciprofloxacin Hydrochloride, Metronidazole or Ciprodiazole® tablets respectively. These solutions were separately heated at 75C° for 4 hours in the dark (to exclude the possible degradative effect of light). The solutions (2 mL) were then transferred to 10 mL volumetric flasks, neutralized by addition of 1mL of 1 mol L^{-1} HCl, and diluted to final volume with distilled water or 0.05 mol L^{-1} edetate disodium respectively [11, 12].

2.8.3. Oxidation

Hydrogen peroxide (H_2O_2; 10%, v/v, 10 mL) was added to 10 mL prepared stock solutions of Ciprofloxacin Hydrochloride and Metronidazole or Ciprodiazole® tablets

respectively. These solutions were separately heated at 75C° for 4 hours in the dark (to exclude the possible degradative effect of light). The solutions (2 mL) obtained were then transferred to 10 mL volumetric flasks and diluted to final volume with distilled water or 0.05 M edetate disodium respectively [11, 12].

2.8.4. Neutral degradation (Thermal degradation)

10 mL of distilled water was added to 10 mL prepared stock solutions of Ciprofloxacin Hydrochloride and Metronidazole or Ciprodiazole® tablets respectively. These solutions were separately heated at 75C° for 4 hours in the dark (to exclude the possible degradative effect of light) to study the effect of thermal stress. Also the experiment was performed on solid-state samples which could be stressed under previous condition and then diluted with a known amount of distilled water. The experiment was performed in the dark to exclude the possible degradative effect of light The solutions (1 mL) obtained were then transferred to 10 mL volumetric flasks and diluted to final volume with distilled water or 0.05 M edetate disodium respectively [11, 12].

2.8.5. Photo stability

10 mL of distilled water was added to 10 mL prepared stock solutions of Ciprofloxacin Hydrochloride and Metronidazole or Ciprodiazole® tablets respectively. These solutions were separately exposed to light providing an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 watt hours/square meter. Also the experiment was performed on solid-state samples which could be stressed under previous condition and then diluted with a known amount of distilled water. The solutions (1 mL) obtained were then transferred to 10 mL volumetric flasks and diluted to final volume with distilled water or 0.05 mol L^{-1} edetate disodium respectively [11, 12].

3. Optimization of chromatographic condition

The ability of a chromatographic method to successfully separate, identify, and quantitate species was determined by several factors which are in the control of the experimenter. Attempting to discover the importance of these factors with respect to the response, design of experiments (DOE) will be utilized to give a powerful suite of statistical methodology which is capable of estimating the effects of each factor in combination as well as alone.

Few trials were carried to determine Ciprofloxacin Hydrochloride and Metronidazole in presence of their main impurities (2-methyl-5-nitroimidazole (Metronidazole imp A), Fluoroquinolonic acid (Ciprofloxacin impurity A), Ciprofloxacin Ethylenediamine (Ciprofloxacin impurity C). Some problems were noticed during early trials using UPLC. These problems are the interference between peaks especially Metronidazole and its impurity A, Ciprofloxacin and its impurity A and decreasing total analysis time.

Firstly, maximum absorption wavelengths for Ciprofloxacin Hydrochloride and Metronidazole were scanned from 400-200 nm under UV as shown in Fig 2. It was found that all drugs have adequate absorption at wavelength 320 nm which was selected for DOE trials.

Low concentration of used buffers (0.01 mol L^{-1} or 0.5%) is adequate for most reversed phase applications. This concentration is also low enough to avoid precipitation problems when significant amounts of organic modifiers are used in the mobile phase [13].

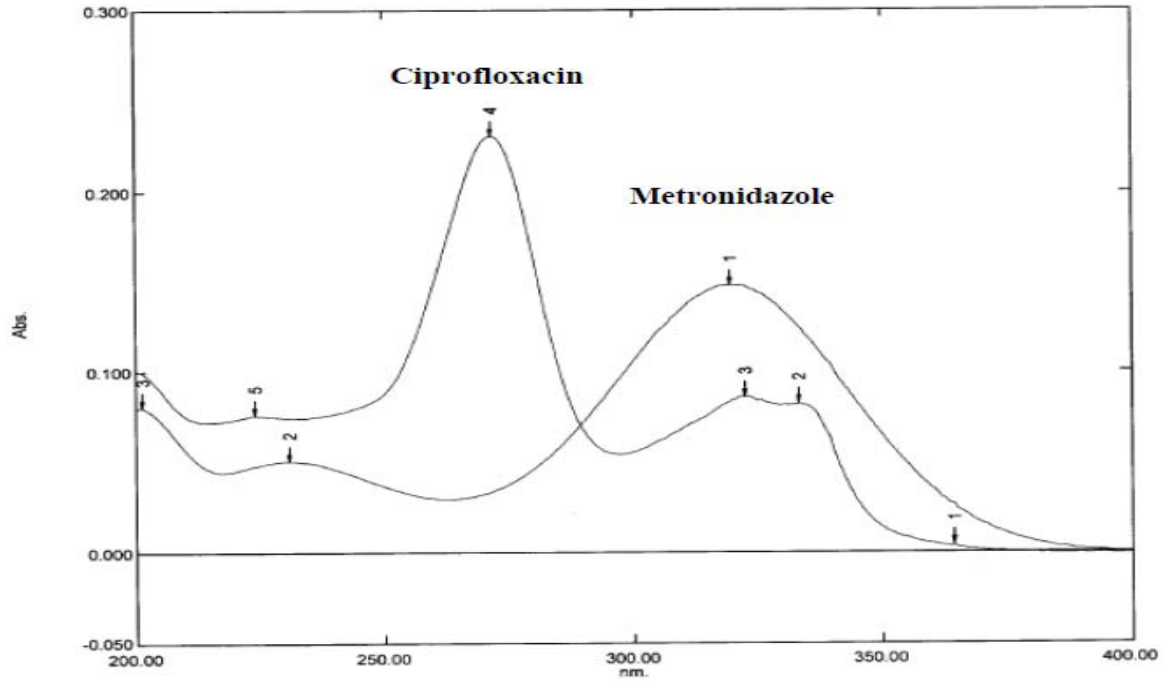


Fig 2: Typical UV spectrum of Ciprofloxacin Hydrochloride and Metronidazole

3.1. Development strategy

Our innovative development strategy follows design of experiment (DOE) principles and can be divided into the five steps: (1) Definition of method goals, (2) risk assessment, (3) design of experiments (4) Design Space, (5) working point selection and verification [14].

3.1.1. Definition of method goals (critical quality attribute)

The primary goal of developing an UPLC stability indicating method is generally to separate the API from impurities (resolution Rs > 2.0) that may impact the quality of the pharmaceutical formulation. From the general equation $Rs = 0.25*N^{1/2}[(\alpha-1/\alpha)]\ (k/1 + k)$ it is obvious that the selectivity parameter has the greatest impact on resolution. Selectivity can be changed by modification of the mobile phase composition, column chemistry and temperature [15]. Other factors such as the need for short analysis times (<10 min) are also considered as critical quality attributes (CQAs) as shown in Table 2. Crucial for the design of experiment approach is to create a visual "Design Space" in which the method is robust.

3.1.3. Risk assessment (critical method parameters)

In an early risk assessment the critical parameters should be identified. That could be method factors which may affect extraction of the compounds of interest in sample preparation (e.g. extraction method, extraction time, extraction solvent) [16] as well as settings in the instrumental analysis. For example the UV spectra of Ciprofloxacin Hydrochloride and Metronidazole were evaluated to select the detection wavelength as mentioned before. Flow rate of mobile phase was 0.6 mL/min and column temperature was 50 C°. Further on the critically influential separation parameters such as stationary phases, type and percent of the eluent A, type and pH of the eluent B were identified and considered as critical method parameters (CMPs) as shown in Table 2 [17].

Table 2: critical method parameters (CMP), critical quality attribute (CQA) and quality method target profile (QMTP)

CMP	Range for each parameter used for DoE		CQA	QMTP
	Low	High		Targeted QTMP
1- Stationary phases	Kinetex C18 (50x4.6 mm, 2.6µm) (Kin 2.6)	Kinetex C18 (50x2.1mm, 1.3µm) (Kin 1.3)	Resolution between Metronidazole imp A and Metronidazole (Res 1)	Resolution should be more than 3
			Resolution between Metronidazole and Ciprofloxacin imp A (Res 2)	Resolution should be more than 4
2- Type of the eluent A	Methanol	Acetonitrile	Resolution between Ciprofloxacin imp A and Ciprofloxacin (Res 3)	Resolution should be more than 2
3- Percent of the eluent A	5:30 in 4 min	5:50 in 4 min	Resolution between Ciprofloxacin and Ciprofloxacin imp C (Res 4)	Resolution should be more than 4
4- Type of the eluent B	0.5%TEA	0.5%PCA	Retention time of Ciprofloxacin imp C (time)	Retention time should be less than 9 min
	0.5%OPA	0.02M HSAS	Theoretical plates (plate)	2000 - 4000
			Symmetry factor (sym)	0.64 - 1.0
			Capacity factor (K)	2 - 10
5- pH of the eluent B	2.5	6.5		

3.1.3. Design of experiments (screening and optimization)

As the result of the risk assessment and identification of CMPs, the five parameters; were screened and optimized using screening design in MODDE software. After all data (CMPs and CQAs) had entered, MODDE software set 19 chromatographic runs to be carried out as shown in Table 3. In this approach the chromatograms obtained by two stationary phases, two type of eluent A, three eluent A compositions, four type of eluent B and 3 pH degrees of eluent B were necessary in order to 4D contour plots (resolution maps) and further on a 4D-sweet spot model (design space) of the critical resolution by using MODDE software as shown in Fig 3 and 4. The ranges between these factors were large enough to induce peak movements to discover hidden peaks and changes in the selectivity as a result of movement of peaks can be studied [18].

3.1.4. Design Space and experimental results evaluation

Design space is the region in which changes to the method parameters will not significantly affect the results. After processing of 19 experimental trials, the resolution between 5 peaks of interest (2-methyl-5-nitroimidazole (Met A), Metronidazole (Met), Fluoroquinolonic acid (Cip A), Ciprofloxacin Hydrochloride (Cip) and Ciprofloxacin Ethylenediamine (Cip C)) and retention time of Ciprofloxacin Ethylenediamine were determined as well as other responses such as capacity factor, symmetry factor and theoretical plate in each chromatogram and input to MODDE software as shown in Table 3.

These experimental results were evaluated using stringent criteria (quality method target profile, QMTP) as shown in Table 2 to create the design space. Design space (shown in green color in Fig 4 a-d) allows alteration of the position of the “working point” without the need for a new validation and a high flexibility in the HPLC/UHPLC laboratory.

The color code in these resolution maps represents the value of the critical resolution, with "green" colors show large resolution values (it conforms quality target method profile (QTMP) as shown in Table 2), yellow, light and dark "blue" colors show low resolution values (it does not conform QTMP) [18].

Table 3: Experimental design (19 runs) obtained by MODDE software for developed method of Ciprodiazole tablet

Exp No	Run Order	Buffer Type	Org Solv Type	Buffer pH	Org Solv %	Column Type	Res 1	Res 2	Res 3	Res 4	Time	K	sym	Plate
1	1	phos	MeoH	2.5	50	Kin1.3	0	10.08	1.49	19.77	5.3	2.94	0.74	855
2	7	tea	MeoH	2.5	50	Kin1.3	0	7.27	1.67	20.96	6.8	1.5	0.75	854
3	17	tea	Acet	6.5	30	Kin1.3	4.38	9.83	1.48	6.86	7	2.77	0.68	812
4	9	hex	Acet	6.5	50	Kin1.3	3.62	21.3	1.59	9.32	4	4.93	0.76	3759
5	10	phos	MeoH	6.5	30	Kin1.3	3.22	9.2	2.42	20.01	6	2.38	0.72	343
6	14	hex	Acet	2.5	30	Kin1.3	5	25	2.29	15	5	2.62	0.84	851
7	18	per	MeoH	6.5	50	Kin1.3	2.72	8.63	1.68	4.56	6	2.09	0.75	866
8	19	per	Acet	2.5	30	Kin1.3	0	19.69	2.91	12	5.4	2.68	0.76	420
9	8	phos	Acet	6.5	30	Kin2.6	7.38	19.09	3.8	10.6	6	5.73	0.6	5086
10	6	hex	MeoH	2.5	30	Kin2.6	4.74	16.37	2.07	10.5	7	4.77	0.56	2388
11	11	per	Acet	6.5	50	Kin2.6	3.87	9.73	2.01	6	7	5.31	0.56	2749
12	12	per	MeoH	2.5	30	Kin2.6	0	27.79	3.29	6.93	8.2	3.16	0.75	2264
13	13	phos	Acet	2.5	50	Kin2.6	0	7.19	1.66	5.7	7.9	4.76	0.6	2256
14	15	tea	Acet	2.5	50	Kin2.6	0	7.28	2.48	5.6	9	4.44	0.83	3580
15	16	tea	MeoH	6.5	30	Kin2.6	4.02	4.13	2.69	6.5	11	4.64	0.65	1783
16	4	hex	MeoH	6.5	50	Kin2.6	3.82	13	0	5.7	9	2.57	0.74	895
17	2	phos	Acet	4.5	40	Kin2.6	3.68	6.45	1.26	5.8	9	2.38	0.75	804
18	3	phos	Acet	4.5	40	Kin2.6	2.17	1.98	1.89	8	9	7.06	0.56	2089
19	5	phos	Acet	4.5	40	Kin2.6	2.87	4.85	1.44	9.52	9	9.42	0.52	2916

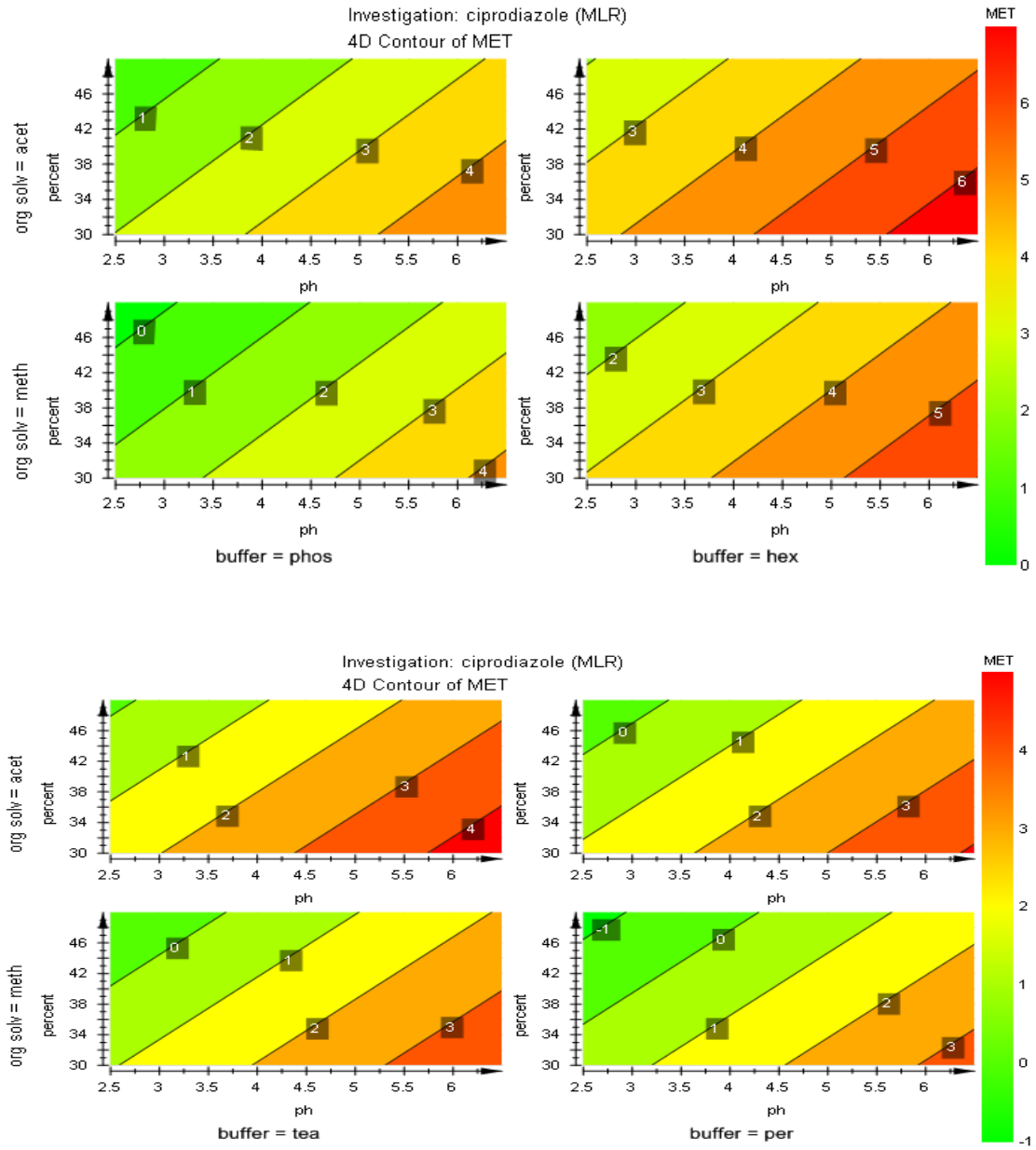


Fig 3a-b: 4D Contour plot (Resolution Map) for resolution between Metronidazole imp A and Metronidazole (Res 1) obtained by MODDE software from 19 DOE experimental run on Phenomenex Kinetex C18 (50x 2.1 mm, 1.3µm)

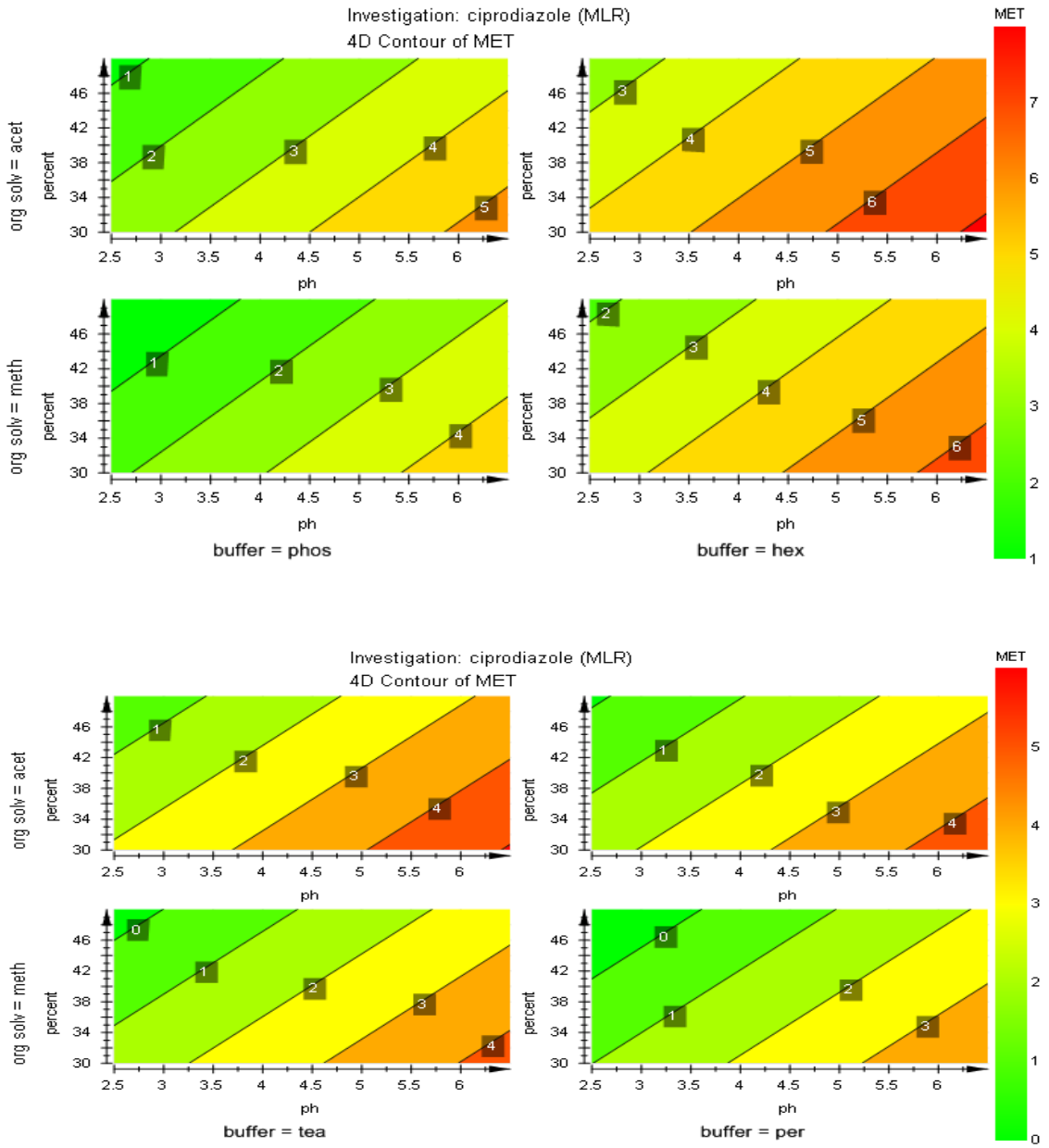
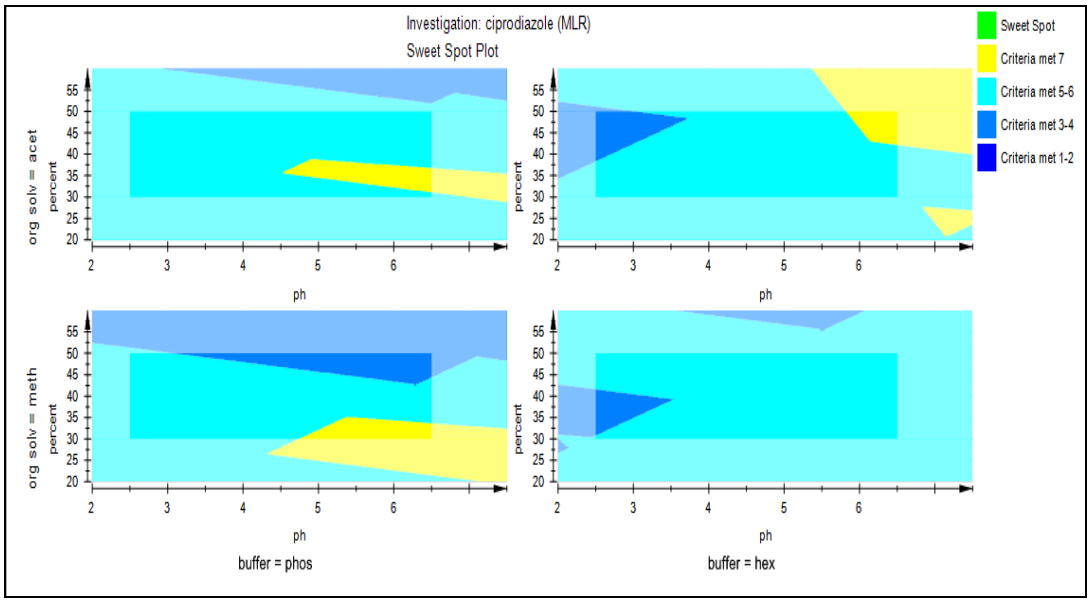


Fig 3c-d: 4D Contour plot (Resolution Map) for resolution between Metronidazole imp A and Metronidazole (Res 1) obtained by MODDE software from 19 DOE experimental run on Phenomenex Kinetex C18 (50x 4.6 mm, 2.6µm)



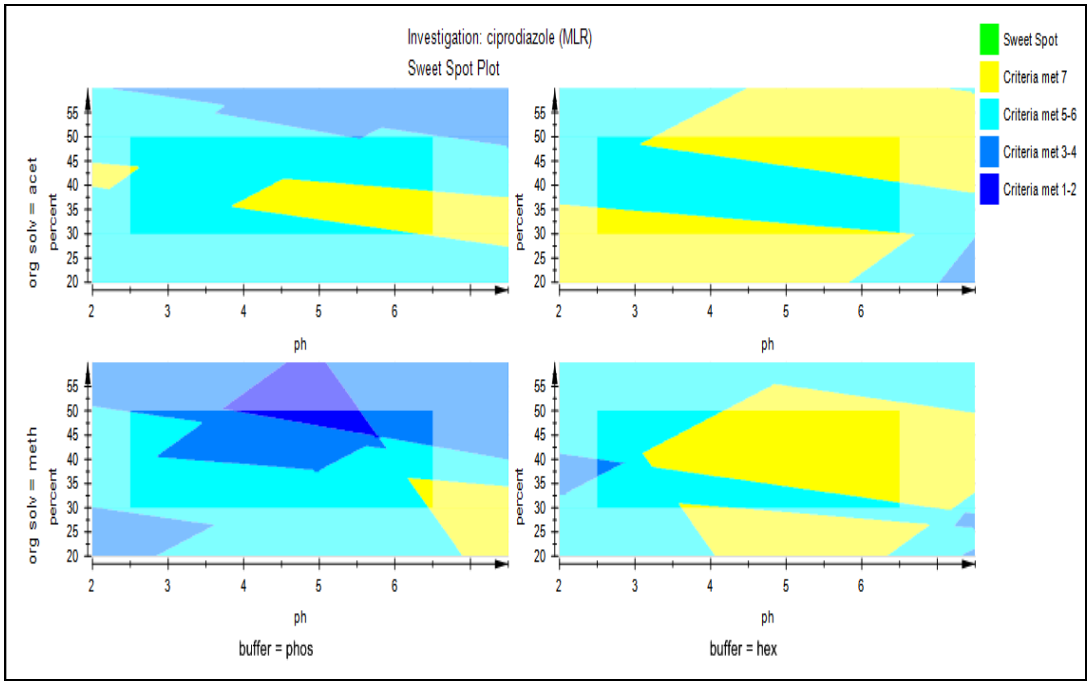
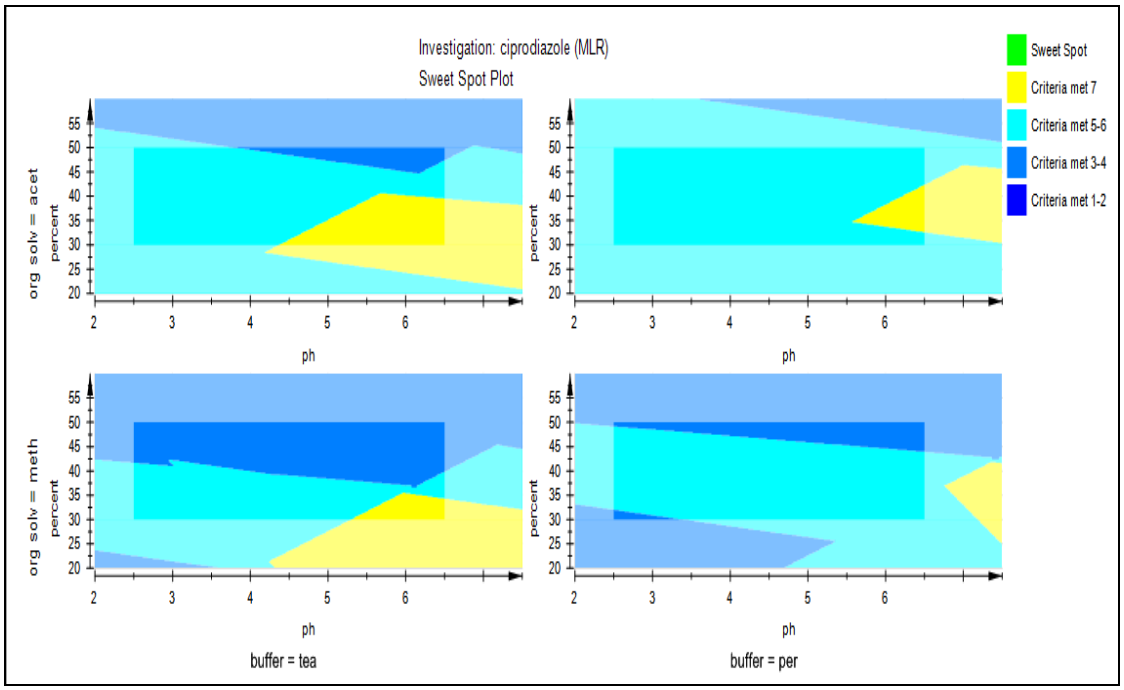


Fig 4a-b: 4D sweet spot plot (Design Space) obtained by MODDE software from 19 DOE experimental run on Phenomenex Kinetex C18 (50x2.1 mm, 1.3µm)



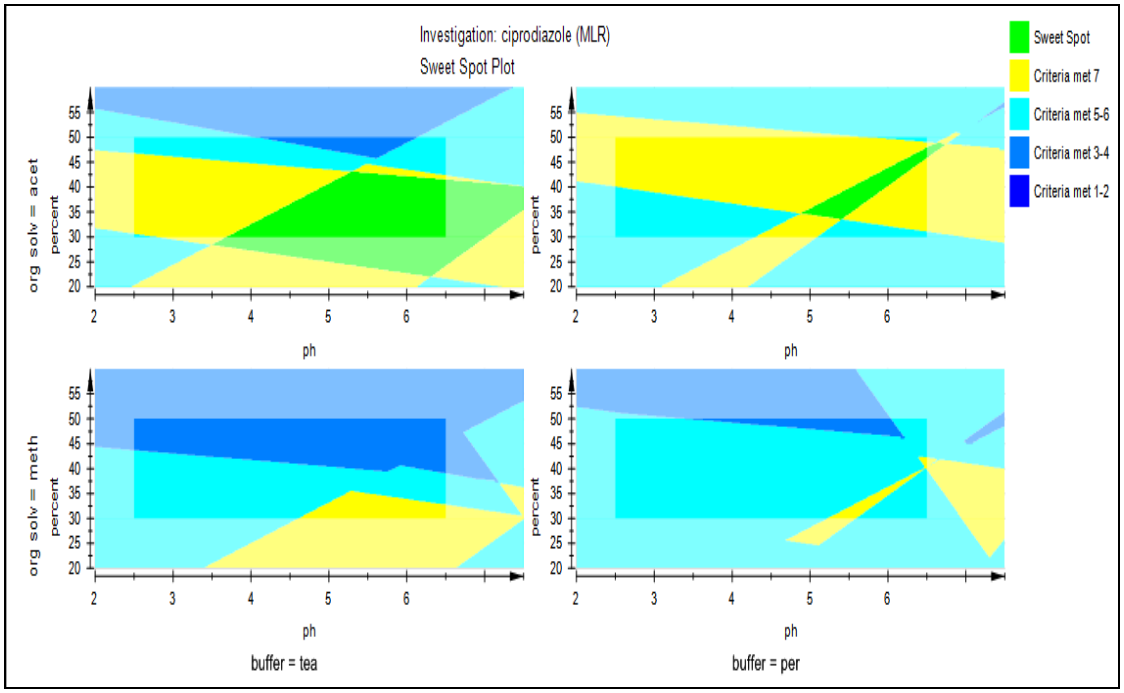


Fig 4c-d: 4D sweet spot plot (Design Space) obtained by MODDE software from 19 DOE experimental run on Phenomenex Kinetex C18 (50x4.6 mm, 2.6µm)

3.2. Working point selection and verification

From the previously constructed design space, the working point was selected by visual examination looking for the highest critical resolution (Rs, crit), best robustness of the method and shorter analysis time. At this point, small changes of CMPs as well as flow rate and dwell volume have no negative influence on the separation of all peaks.

3.2.1. Selection of proper pH

This working point was found in the sweet spot plot as mentioned under chromatographic conditions except pH of aqueous phase (triethylamine, TEA 0.5%) nm). It was selected to be 6.5 but peak tailing of ciprofloxacin and its imp A was showed as shown in Fig 5. So, another point in the design space i.e. another pH (4.5) was selected so, tailing problem disappeared.

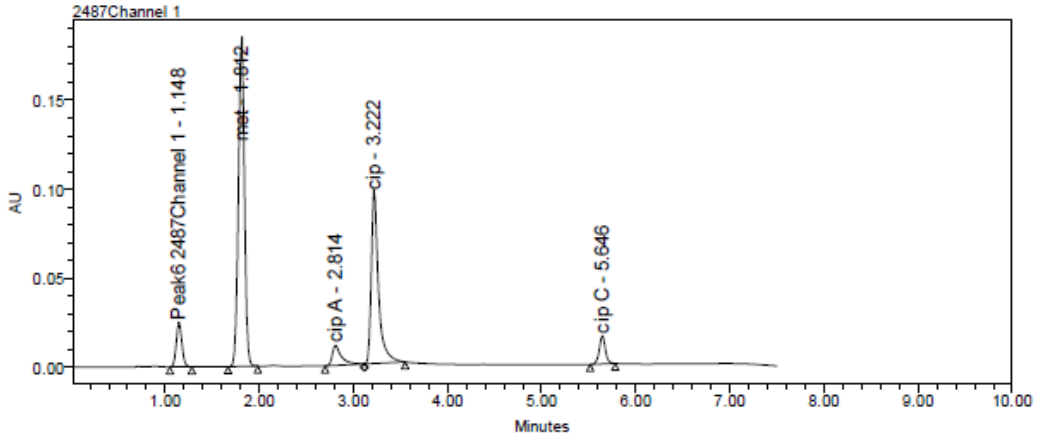


Fig 5: Typical UPLC chromatograms under optimized chromatographic conditions at pH 6.5 shows peak tailing of ciprofloxacin and its imp A.

3.2.2. Selection of proper solvent

Optimized method was verified by method validation especially method robustness as mentioned above according to ICH guideline Q2 (R1) [19]. During validation, another problem was appeared in accuracy (recovery results) of Ciprofloxacin and assay of it in Ciprodiazole tablets due to chelating between ciprofloxacin and magnesium stearate which led to 20% bias in recovery result of ciprofloxacin. Organic solvents were tried to solve this problem but acetonitrile could not dissolve Ciprofloxacin and methanol produced broad bad peak of Metronidazole as shown in Fig 6. Diluted hydrochloric acid (0.1 and 0.2 M) was also tried and produced the same peak shape as methanol. After 0.05M Edetate disodium had been tried, it produced good recovery results (accuracy) of Ciprofloxacin and good symmetric peak shape of Metronidazole as shown in Fig 7. Finally, after chromatographic conditions had been optimized and proper solvent had been selected, the final chromatogram of Ciprofloxacin, Metronidazole and their impurities was achieved as shown Fig 8a.

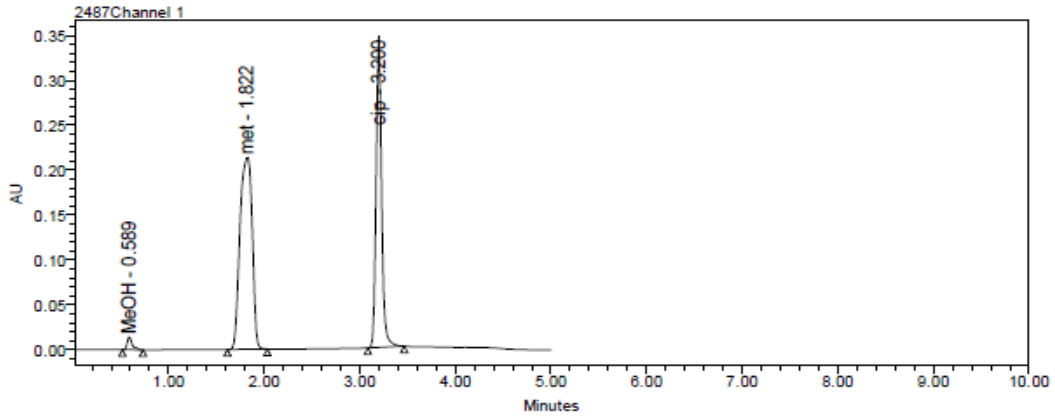


Fig 6: Typical UPLC chromatograms of Ciprodiazole® tablets under optimized chromatographic conditions by using methanol as a solvent which produced a bad broad peak of Metronidazole.

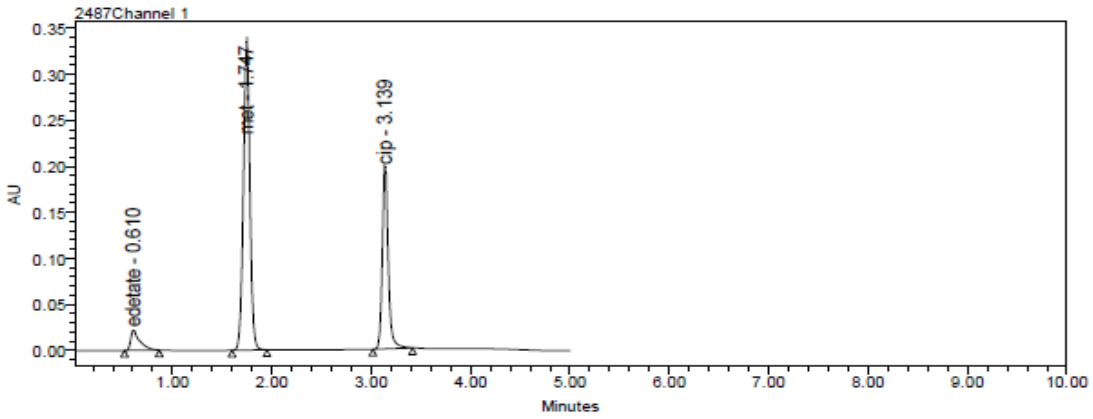
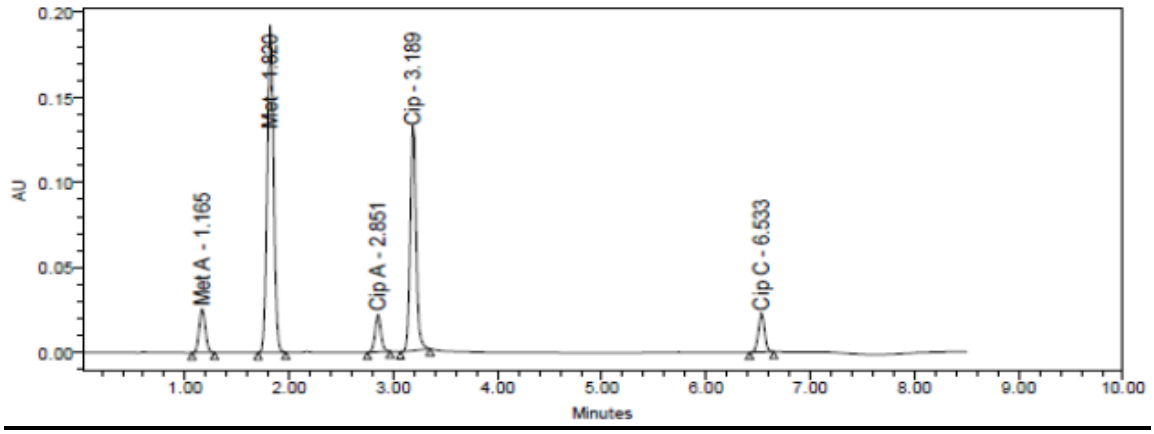
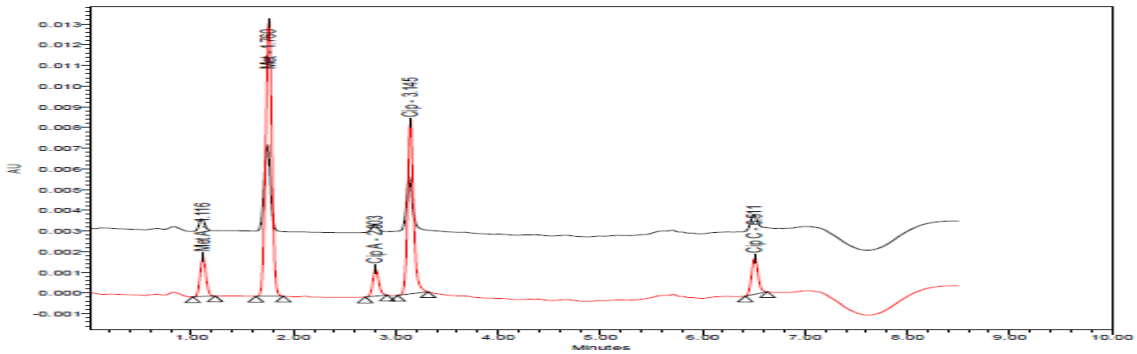


Fig 7: Typical UPLC chromatograms of Ciprodiazole® tablets under optimized chromatographic conditions by using 0.05 M Edetate disodium as a solvent which produced a good peak shape of Metronidazole.



(A)



(B)

Fig 8: (A, B) Typical UPLC chromatograms obtained from 2 µl injections of 2-methyl-5-nitroimidazole (Met A), Metronidazole (Met), Fluoroquinolonic acid (Cip A), Ciprofloxacin Hydrochloride (Cip), Ciprofloxacin Ethylenediamine (Cip C) respectively under optimized chromatographic conditions. (A) At 100% conc. Level of all compounds and tablet. (B) At DL and QL

4. Results and discussion

4.1. Method validation

4.1.1. Specificity

A placebo for Ciprodiazole® tablet was prepared by mixing its excipients such as pregelatinized starch, sodium starch glycolate, P.V.P K 30, lactose monohydrate, sodium lauryl sulphate, magnesium stearate. Solutions were prepared by following the procedure described in the section on sample preparation. The commonly used tablet excipients did not interfere with the method as shown in Fig 8A. The resulted chromatogram shows that the tablet excipients have negligible contribution after the void volume at the method detection wavelength of 320 nm. The method were also evaluated by assessing whether impurities such as (2-methyl-5-nitroimidazole, Fluoroquinolonic acid and Ciprofloxacin Ethylenediamine) and degradation products present in the pharmaceutical formulations - obtained from stress studies involving acid, base, peroxide, and heat stored under ICH stability conditions - interfered with the analysis of Ciprofloxacin Hydrochloride and Metronidazole as shown in Fig 8A [19].

4.1.2. Linearity and range

Five concentrations were chosen in the ranges (1.25 - 75 μgmL^{-1}) for corresponding levels of 2.5-150% w/w of the nominal analytical concentration of Ciprofloxacin Hydrochloride and Metronidazole.

The linearity of peak area responses versus concentrations was demonstrated by linear least square regression analysis. The linear regression equations were { Y = 18471 X -17237 (r= 0.9994) and Y = Y = 31689 X -18709 (r= 0.9998} for Ciprofloxacin Hydrochloride and Metronidazole respectively as shown in Table 4. Where Y is the peak area of standard solution and X is the drug concentration [19].

Table 4: Calibration data was resulted from method validation of Ciprofloxacin Hydrochloride and Metronidazole respectively

Item	Ciprofloxacin Hydrochloride	Metronidazole
Linear range (μgmL^{-1})	1.25-75	1.25-75
Detection limit (μgmL^{-1})	0.18	0.18
Quantitation limit (μgmL^{-1})	0.56	0.53
Regression data		
No.	10	10
slope (b)	18471	31689
Standard deviation of the slope	1870.98	31.953
intercept (a)	-17237	-18709
Standard deviation of the intercept	962.8	1677.2
correlation coefficient ®	0.9994	0.9998
Standard error of regression	15.863	17.064

(Y = a + bC, where C is the concentration of the compound (µg/mL) and Y is the drug peak area)

4.1.3. Precision

The precision of the assay was investigated by measurement of both repeatability and intermediate precision.

4.1.3.1. Repeatability

Repeatability was investigated by injecting a minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each) and percentage SD was calculated in Table 5.

Table 5: Repeatability and Intermediate precision and Accuracy (*Recovery %)* of Ciprofloxacin Hydrochloride and Metronidazole respectively

Drug Name	Conc.	Ciprofloxacin		Metronidazole	
		AV±SD μgmL^{-1}	AV±SD %	AV±SD µgmL-1	AV±SD %
Repeat-ability	50%	24.67±0.13	98.69±0.51%	25.04±0.12	100.16±0.50%
	100%	50.54±0.11	101.08±0.21%	49.59±0.12	99.18±0.25%
	150%	76.52±0.12	102.03±0.16%	74.74±0.16	99.66±0.21%
Intermediate precision	50%	25.00±0.39	100.01±1.55%	25.26±0.30	101.04±1.20%
	100%	51.00±0.67	102.01±1.33%	50.06±0.53	100.11±1.06%
	150%	75.07±1.64	100.09±2.19%	74.93±0.50	99.91±0.67%
Accuracy	75%	37.97±0.29	101.25±0.78%	38.12±0.07	101.65±0.19%
	100%	50.18±0.16	100.36±0.32%	50.16±0.09	100.32±0.19%
	125%	63.23±0.25	101.18±0.40%	63.07±0.20	100.91±0.32%

N.B. (50%, 75%, 100%, 125% and 150%) Concentration of Ciprofloxacin Hydrochloride and Metronidazole are 25, 37.5, 50, 62.5 and 75µg/ml respectively.

4.1.3.2. Intermediate precision

In the inter-day studies, standard and sample solutions prepared as described above, were analyzed in triplicate on three consecutive days at specified range for the procedure (e.g., 3 concentrations/3 replicates each) of the test concentration and percentage SD was calculated in Table 5 [19].

4.1.4. Accuracy

Accuracy was assessed using 9 determinations over 3 concentration levels covering the specified range (50,100 and 150%). Accuracy was reported in Table 5 as percent recovery by the assay of known added amount of analyte in the sample [19].

4.1.5. Limits of detection and Limits of quantitation

According to the ICH recommendations, determination of limits of detection and quantitation was based on the standard deviation of the y-intercepts of regression lines (n=3) and the slope of the calibration plots as shown in Fig 8B and Table 4 [19].

4.1.6. System suitability tests

System suitability tests were used to verify that the resolution and reproducibility were adequate for the performed analysis. The system suitability tests included number of theoretical plates, resolution, peak tailing, capacity factor and selectivity factor. Results are revealed in Table 6.

4.1.7. Robustness

Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small variations in method parameters and provides an indication of its reliability during normal usage [19]. Robustness was tested by studying the effect of changing

in mobile phase pH by ± 0.2, the percentage of organic solvent (acetonitrile) in the mobile phase by ± 2 %, temperature by ± 5 Cº, wavelengths by ± 2 nm and flow rate by ± 0.05 mL/min. all previous small variation had no significant effect on the chromatographic resolution of between studied drugs and their impurities as shown in Table 7.

Table 6: System suitability parameters of all drugs were obtained from Method Validation

Drugs/Parameters	Theoretical Plates (N)	Resolution (R)	Capacity Factor (K)	Tailing Factor (T)	Selectivity (α)
2-methyl-4-nitroimidazole (Met A)	1812.05	-	1.33	1.08	-
Metronidazole (Met)	3970.49	5.96	2.64	1.03	1.98
Fluoroquinolonic acid (Cip A)	12774.33	9.78	4.70	1.06	1.78
Ciprofloxacin Hydrochloride (Cip)	15377.78	3.39	5.38	1.08	1.14
Ciprofloxacin Ethylenediamine (Cip C)	58934.6	32.5	12.07	0.96	2.24

Table 7: Effect of Changes of Some Parameters on Resolution during Method Robustness

Parameters	Flow Rate		PH		Acetonitrile %		Wavelength		Temperature	
Value	0.55	0.65	4.3	4.7	3	7	318	322	48	52
2-methyl-4-nitroimidazole (Met A)	-	-	-	-	-	-	-	-	-	-
Metronidazole (Met)	7.02	5.5	6.44	6.66	8.16	4.90	6.56	6.17	6.56	5.73
Fluoroquinolonic acid (Cip A)	7.88	9.51	11.50	11.11	8.40	9.78	7.87	11.03	10.73	10.44
Ciprofloxacin Hydrochloride (Cip)	3.62	3.57	3.76	3.83	3.87	3.69	3.75	3.80	3.86	3.86
Ciprofloxacin Ethylenediamine (Cip C)	33.85	33.81	35.56	33.68	32.98	35.93	34.80	35.28	34.76	34.85

4.1.8. Stability of analytical solution

As a part of evaluation of robustness, solution stability was evaluated by monitoring the peak area response. Standard stock solutions in 0.05 M edetate disodium were analyzed right after its preparation 1, 2 and 3 days after at room temperature. The change in standard solution peak area response over 3 days was (2.30 and 0.91 %) for Ciprofloxacin Hydrochloride and Metronidazole respectively. Their solutions were found to be stable for 3 days at room temperature at least.

4.2. Application on pharmaceutical Preparation

The proposed methods were successfully used to determine Ciprofloxacin Hydrochloride and Metronidazole respectively in Ciprodiazole® tablet. Five replicate determinations were performed. Satisfactory results were obtained for each compound in good agreement with label claims. The results obtained were compared statistically with those from the published method [8] by using Student's t-test and the variance ratio F-test. The results showed that the t and F values were smaller than the critical values as shown in Table 8. So, there were no significant differences between the results obtained from this method and published methods as shown in Table 8.

Table 8: Statistical comparison of the proposed and published methods for determination of Ciprofloxacin Hydrochloride and Metronidazole respectively in their dosage forms by reported method (T- student test) and (F –test for variance)

Drug name	Recovery ± SD		Calculated t- values	Calculated F- values
	Proposed methods	Reference method		
Ciprofloxacin Hydrochloride	101.06±0.59	102.92±1.69	2.16	0.12
Metronidazole	101.32±0.86	100.34±1.38	1.83	0.381

(Where the Tabulated t-values and F -ratios at p = 0.05 are 2.57 and 5.05)

4.3. Forced degradation studies

Ciprofloxacin Hydrochloride, Metronidazole and Ciprodiazole® tablets bulk powders were subjected to various stress condition. Ciprofloxacin Hydrochloride exhibited low percentage of degradation about 6% under acidic and basic which is smaller than degradation percentage under oxidative and photolytic conditions about 23 % and 30% as shown in Table 9 and Fig 9-13. The main degradations of Ciprofloxacin Hydrochloride are Fluoroquinolonic acid (Cip A) and Ciprofloxacin Ethylenediamine (Cip C). Metronidazole was exhibited 10 % degradation under thermal and oxidative conditions which is smaller than degradation percentage under acidic and photolytic conditions about 20 % respectively and it was fully degraded under basic conditions as shown in Table 9 and Fig 9-13. The main degradation of Metronidazole is 2-methyl-5-nitroimidazole (Met A).

Table 9: Results of degradation percent of Ciprofloxacin Hydrochloride and Metronidazole obtained from stress test condition

Stress test condition	Metronidazole		Ciprofloxacin Hydrochloride	
	Peak Area	Assay after Degradation %	Peak Area	Assay after Degradation %
Std	632940.00	100.00	366520.00	100.00
HCl (1M) at 75 ºC / 6 hours	495767.93	78.33	348039.29	94.96
NaOH (1M) at 75 ºC / 6 hours	0.00	0.00	352330.36	96.13
H_2O_2 (3%) at 75 ºC / 6 hours	550525.95	86.98	284798.66	77.70
Heat at 75 ºC at 75 ºC / 6 hours	583609.98	92.21	231360.39	63.12
Light	490033.43	77.42	256441.07	69.97

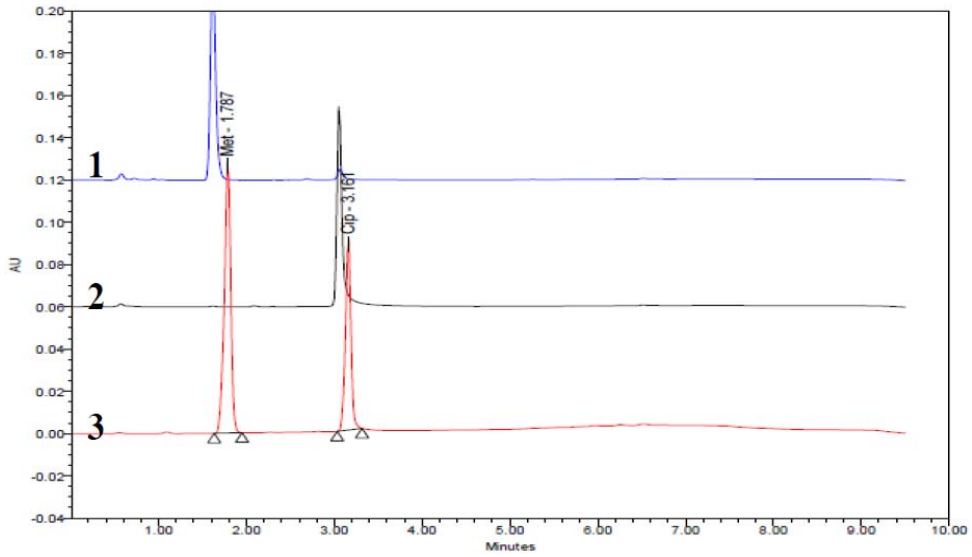


Fig 9: Typical UPLC chromatograms obtained from 20 µl injections of solutions of Metronidazole, Ciprofloxacin Hydrochloride and Ciprodiazole® tablets bulk powders which were subjected to acidic condition under optimized chromatographic conditions. (Descending order)

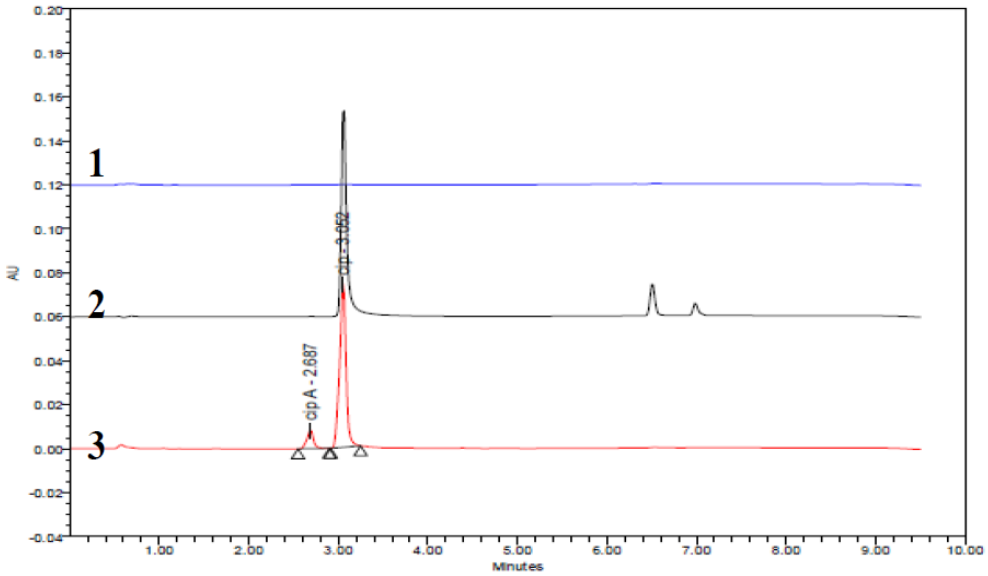


Fig 10: Typical UPLC chromatograms obtained from 20 µl injections of solutions of Metronidazole, Ciprofloxacin Hydrochloride and Ciprodiazole® tablets bulk powders which were subjected to alkaline condition under optimized chromatographic conditions. (Descending order)

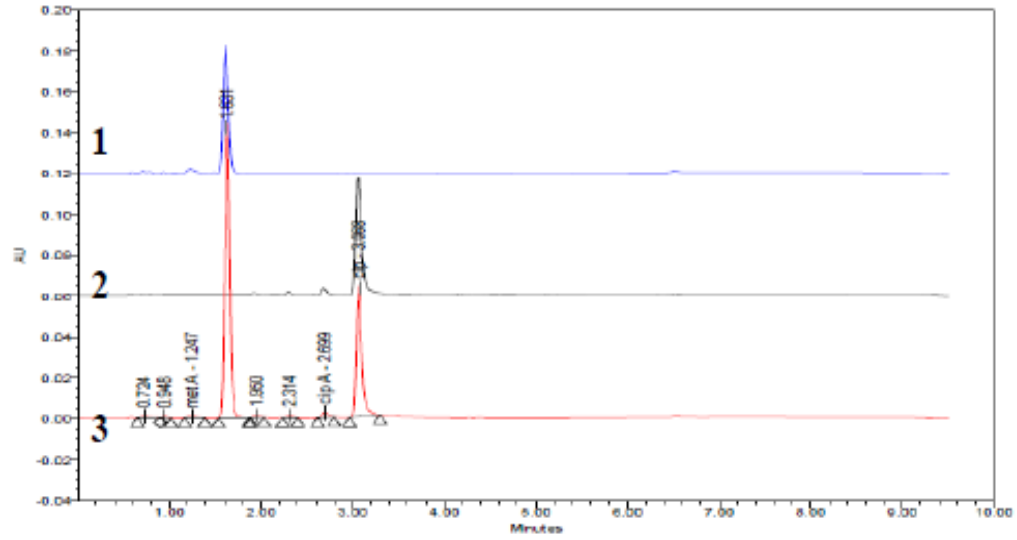


Fig 11: Typical UPLC chromatograms obtained from 20 µl injections of solutions of Metronidazole, Ciprofloxacin Hydrochloride and Ciprodiazole® tablets bulk powders which were subjected to oxidative condition under optimized chromatographic conditions. (Descending order)

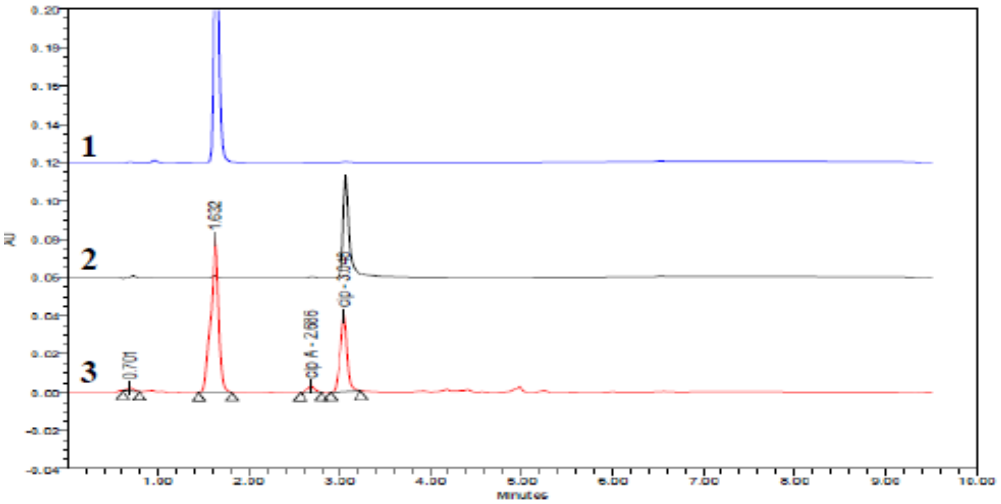


Fig 12: Typical UPLC chromatograms obtained from 20 µl injections of solutions of Metronidazole, Ciprofloxacin Hydrochloride and Ciprodiazole® tablets bulk powders which were subjected to thermal condition under optimized chromatographic conditions. (Descending order)

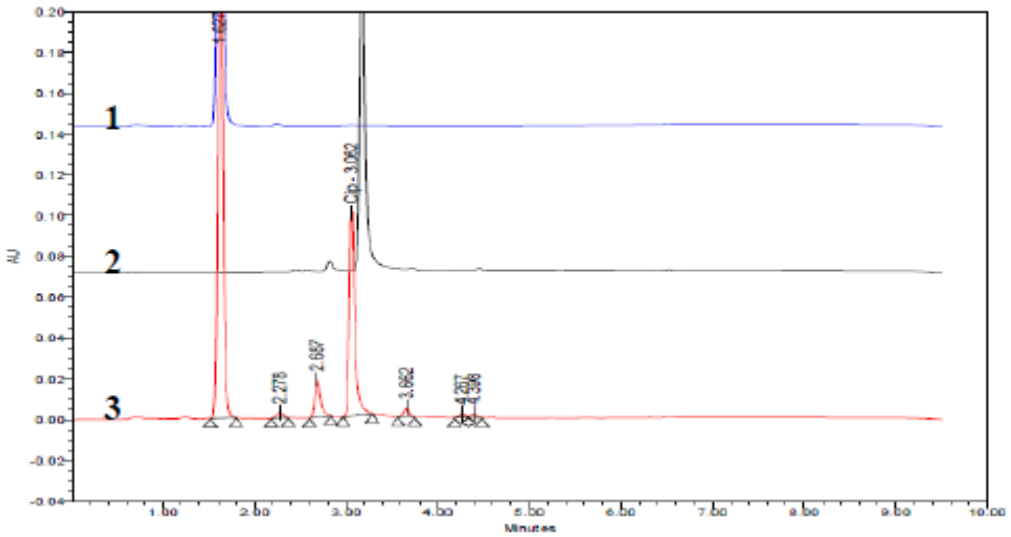


Fig 13: Typical UPLC chromatograms obtained from 20 µl injections of solutions of Metronidazole, Ciprofloxacin Hydrochloride and Ciprodiazole® tablets bulk powders which were subjected to photolytic degradation under optimized chromatographic conditions. (Descending order)

4.4. Discussion

New developed method has several merits than other published methods in literature. It utilized DOE technique in development and optimization which led to a high robust method. It determines Ciprofloxacin Hydrochloride and Metronidazole in presence of its degradation products in a single run and it can be used as a stability indicating method. Good peaks shape and resolution between studied drugs. It was performed by UPLC technique which led to shorter retention time (7 minutes). Specificity was proven clearly after degradation products of both drugs had been separated.

5. Conclusion

A simple, accurate, precise, robust and reliable LC method has been established for simultaneous determination for Ciprofloxacin Hydrochloride and Metronidazole respectively in tablets.

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