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Original Article

NOVEL ISOCRATIC REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF CIPROFLOXACIN AND PHENAZOPYRIDINE IN SOLID DOSAGE FORM

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ABSTRACT

Objective: To develop a new, simple, validated reverse phase high performance liquid chromatography (RP-HPLC) method for the simultaneous quantitative determination of Ciprofloxacin (CFX) and Phenazopyridine (PZD) in combined tablet dosage form.

Methods: Simultaneous chromatographic separation and quantification of CFX and PZD was achieved using waters Alliance HPLC system on a C_{18} column (250 x 4.6 mm i. d, 5 µm particle size) maintained at ambient temperature in isocratic mode, with mobile phase comprising of ammonium ortho phosphate (0.01 N, pH 3.5 adjusted with dilute ammonia solution) and acetonitrile at the ratio of 50:50 (v/v) pumped on to the column at a flow rate of 1 ml/min followed by detection of eluents at isoabsorptivity wavelength of 275 nm within total run time was 8 min. Water and Acetonitrile in the ratio of 90:10 (v/v) were used as needle wash and the injection volume was 20 µl.

Results: CFX and PZD have eluted with retention time (tr) of 2.783 min & 4.111 min, respectively and quantification permitted over a linear concentration range of 25-150 µg/ml, (R^2 =1, Y= 23106 x+5020) & 10-60 µg/ml, (R^2 =1, Y= 35116 x-900.36) respectively. The limits of detection and of quantification were 1.44 & 4.38 µg/ml for CFX and 0.964 & 2.92 µg/ml for PZD, respectively. % mean recoveries were ranging 99.29-100.57 % for CFX and 99.36-100.02 % for PZD respectively while, the relative standard deviation (% RSD) of intra-day and inter-day precision was 0.91 & 0.79, for CFX and 1.03 & 0.83, for PZD respectively. The specificity data of the proposed method indicated that excipients in the formulation did not interfere with the drug peaks of CFX and PZD. Furthermore, the well-shaped peaks buttressed the specificity of the method.

Conclusion: The RP-HPLC method is simple, cost-effective and accurate for the simultaneous estimation of CFX and PZD in both bulk and pharmaceutical dosage form and it can be employed for routine laboratory analysis.

Keywords: Ciprofloxacin, Phenazopyridine, RP-HPLC, Isocratic, Pharmaceutical dosage forms, Simultaneous Analysis, Validation.

INTRODUCTION

Ciprofloxacin (CFX) is a second-generation fluoroquinolone [1, 2], and chemically. It is 1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid [3, 4]. It is a faintly yellowish to light yellow crystalline substance with molecular formula $C_{17}H_{18}FN_3O_3$ and its molecular weight is 331.4. It is used alone or in combination with other antibacterial drugs in the empiric treatment of infections for which the bacterial pathogen has not been identified, including urinary tract infections [5] and abdominal infections [6]. It kills bacteria by interfering with the enzymes that cause DNA to rewind after being copied, which stops synthesis of DNA and of protein. CFX is official in Indian Pharmacopoeia (IP), British Pharmacopoeia (BP), United States Pharmacopoeia (USP) and IP [7], BP [8] and USP [9] describe HPLC methods for the estimation of CFX alone. There are several analytical methods were reported for the determination of CFX individually using flow injection analysis [10], spectrofluorimetric method [11], hydrotrophy technique [12], HPLC [13], biological fluids [14-16], human gingival crevicular fluid [17], spectrophotometry [18], and in combination with other therapeutic agents using spectrophotometry [19-26], HPLC [27, 28], UPLC [29, 30], were reported in the literature.

Phenazopyridine (PZD) is a urinary tract analgesic, and chemically, it is 3-phenyldiazenylpyridine-2, 6-diamine. It is used for relieving pain, burning, urgency, frequent urination, and discomfort caused by irritation of the lower urinary tract mucosa caused by infection, trauma, surgery, endoscopic procedures, and the passage of sounds or catheters. It is sometimes used in conjunction with an antibiotic or other anti-infective medication at the beginning of treatment to help provide immediate symptomatic relief. Phenazopyridine does not treat infections or injury and it is only used for symptom relief. Exactly how phenazopyridine works is not known. It is thought to work by relieving pain on the lining of the urinary tract. Phenazopyridine HCl is official in United States Pharmacopoeia (USP) [31] describe HPLC method for its estimation. Estimation of Phenazopyridine individually and combination with other therapeutic agent in biological fluids [32-36], spectrophotometric [37] & HPLC [38-40] were reported in literature. The combination of ciprofloxacin and phenazopyridine is very useful in the treatment of urinary tract infections. Chemical structures of CFX and PZD were shown in fig. 1(a) and 1 (b).

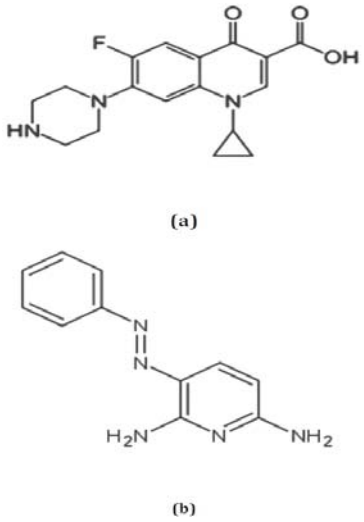

Fig. 1: Chemical structure of (a) CFX and (b) PZD

On literature survey, it was found that no method could be found for the simultaneous estimation of Ciprofloxacin and Phenazopyridine in combined dosage forms and no method is available in the pharmacopoeias. In view of the need for a suitable validated [41] methods for routine analysis of combined formulations, attempts are being made to develop simple, novel (new), precise and accurate analytical method for simultaneous estimation of titled ingredients and extend it for their determination in the formulation.

The development of HPLC methods for the determination of drugs has received considerable attention in recent years because of their importance in the quality control of drugs and drug products. The main objective of developing HPLC methods for pharmaceuticals to determine the active medicament in terms of quality and quantity which is essential to ensure the therapeutic efficacy. In order to improve the patient compliance during drug therapy there is a need to develop and validate suitable analytical methods for the pharmaceuticals and no matter whether the therapeutic agent is present as individual or else in the combination with other drugs, the amount of active ingredient and purity at individual should comply with posology direction in order to achieve effective treatment. The goal of this study was to develop and validate a RP-HPLC method for the simultaneous estimation of CFX and PZD in bulk and pharmaceutical commercial preparations.

MATERIALS AND METHODS

Instrumentation

Separation was performed on Water's Alliance HPLC system 2695 consisting of a quaternary, low-pressure mixing pump and inline vacuum degassing. The auto sampler has a maximum capacity of 120 vials (12 x 32, 2 ml) with programmable temperature control from 4 to 40 °C. A heated column compartment provides temperatures from 5 ^{0}C above ambient to 65 °C. The detector is a photodiode array (model 2996) with a wavelength range of 190-800 nm and sensitivity settings from 0.0001-2.0000 absorbance units. All components of the HPLC are controlled through Waters Empower software.

Materials

Standards of CFX (99.5 %) and PZD (99.3 %) were obtained as gift samples from a reputed pharmaceutical company and pharmaceutical product of CFX and PZD tablet (Vadelon) with a label claim of 250 mg and 100 mg, respectively was procured from local pharmacy. HPLC grade water (Merck) & Acetonitrile (Qualigens), Ammonium Ortho phosphate (SD fine chemicals limited, Mumbai, India), Electronic analytical balance (Sartorius), Micro pipette (In labs, 10-100 µl), pH meter (Elico) & Desiccator were employed in the study.

Chromatographic conditions

Isocratic elution of mobile phase comprising of 0.01 N Ammonium orthophosphate and Acetonitrile in the proportions of 50:50 V/V with a flow rate of 1 ml/min was performed on a C_{18} column (250x 4.6 mm i. d; 5 µm). The run time was set at 8 min and the column temperature was maintained at 30^{0}C. The volume of injection was 20 µl, prior to injection of analyte, the column was equilibrated for 30-40 min with mobile phase. The eluents were monitored at 275 nm using PDA detector. Data was acquired, stored and analysed with water's Empower software. The mobile phase was premixed, filtered through a 0.45 µm nylon filter and degassed by sonication.

Preparation of mobile phase

Buffer [0.01 N Ammonium Ortho Phosphate] and HPLC grade solvent of acetonitrile were used for the preparation of the mobile phase in a ratio of 50:50 (v/v). The contents of the mobile phase were filtered before use through a 0.45 µm membrane filter, sonicated and pumped from the solvent reservoir to the column at a flow rate of 1 ml/min.

Preparation of standard solutions

A stock solution containing CFX and PZD was prepared by dissolving 250 mg of CFX and 100 mg of PZD in a 100 ml volumetric flask containing 60 ml of diluent, then made up to volume with diluent. Daily working standard solutions of CFX & PZD was prepared by suitable dilution of the stock solution in the mobile phase. Six standards of the drug solution were prepared in the mobile phase containing CFX & PZD at a concentration of 25-150 &10-60 µg/ml, respectively. Each of these drug solutions (20 µl) was injected six times into the column, the peak area and retention times were recorded.

Procedure for pharmaceutical formulation

To determine the CFX & PZD contents of the tablet formulation, twenty tablets of VADELON labelled to contain 250 mg & 100 mg of CFX & PZD were weighed to determine the average weight of the tablets, and then crushed, mixed using a mortar and pestle. A sample of the powder equivalent to 250 mg & 100 mg of CFX & PZD was accurately weighed, mixed with known amount of mobile phase and the active pharmaceutical ingredients was extracted by vortex mixing followed by ultrasonication and then filtered through a 0.45 µm membrane filter. The solution was diluted suitably with the mobile phase to obtain expected concentrations of 100 and 30 µg/ml for CFX & PZD, respectively. Prepared drug solutions (20 µl) were injected six times into the column, the peak area and retention times were recorded.

RESULTS AND DISCUSSION

Method development

A simple, rapid, economic RP-HPLC method has been developed for simultaneous estimation of CFX & PZD bulk and pharmaceutical dosage forms. The method was optimized to provide a good separation of the components (acceptable theoretical plates and resolution between peaks) with sufficient sensitivity and suitable peak symmetry (peak tailing factor<2) in a short run. For this purpose, the analytical column, solvent selection, mobile phase composition, flow rate, and detector wavelength were studied. The use of hydrophobic stationary phases usually provides adequate retention of organic non polar molecules. The chromatographic separation was achieved using an RP-C_{18} column because it was suitable for separation of CFX & PZD with adequate resolution and gave symmetrical peak shapes. Our experiments and data reported in the literature showed that both the methanol and acetonitrile could be used an organic modifier in the mobile phase. The use of acetonitrile as a mobile phase organic modifier resulted in better sensitivity compared to methanol. Tests involving the use of mixtures of acetonitrile and different buffer solutions (e. g., potassium phosphate or ammonium acetate) was made to optimize the mobile phase with different pH values, finally 0.01 N Ammonium orthophosphate and Acetonitrile in the proportions of 50:50 (v/v) was selected as mobile phase whose combination given good peak symmetry & sensitivity. Our experiments revealed that isocratic elution with simple mobile phase were given good results than gradient with complicated mobile phases. The method has many advantages, e. g., simplicity, isocratic conditions, and less flow rate, inexpensive mobile phases. Under these conditions, the retention times of CFX & PZD were about 2.783 min & 4.111 min, respectively with a good peak shape (peak symmetry), and the chromatographic analysis time was 8 min.

Method validation

The method was validated as per ICH guidelines for validation of analytical procedures for different validation parameters. The method was validated for its specificity, linearity, accuracy, precision, selectivity, robustness, ruggedness, LOD and LOQ. A system suitability test was also carried out to evaluate the reproducibility of the analytical system using five replicate injections of a reference solution.

Specificity

The results from the stress testing studies indicated the method was highly specific for CFX & PZD. Based on peak purity data of CFX & PZD, every compound showed that the peaks were homogeneous and there were no co-eluting peaks indicating that the method was specific.

Linearity

The linearity was evaluated by linear regression analysis by the least-squares regression method, which was used to calculate the r-value, *y*-intercept, and slope of the regression line. Three analytical curves were constructed by plotting peak areas against the respective concentrations. From the stock reference solutions of CFX & PZD, six concentrations were prepared in the mobile phase in the range of 25–150 µg/ml & 10-60 µg/ml, respectively and those were found to be linear with a correlation coefficient (r^2) of 1 & 1, the corresponding linear regression equation being y = 23106 x+5020 & y = 35116 x-900.36. The data of linearity were shown in table-1 & 2 and corresponding linearity curves were shown fig 2 & 3.

Table 1: Linearity of CFX

S. No.	Concentration (µg/ml)	Peak area*
1.	25	581410
2.	50	1168769
3.	75	1735255
4.	100	2322825
5.	125	2889882
6.	150	3467801

*Mean of six values (r^2= 1; slope= 23106; intercept= 5020)

Table 2: Linearity of PZD

S. No.	Concentration (µg/ml)	Peak area*
1.	10	348938
2.	20	704915
3.	30	1046775
4.	40	1406389
5.	50	1754362
6.	60	2106752

*Mean of six values (r^2= 1; slope= 35116; intercept=-900.36)

Precision of the method

Precision is the measure of how close the data values to each other for a number of measurements under the same analytical conditions (Repeatability). System precision of the method was evaluated by performing six replicate measurements/injections of standard preparation and method precision was evaluated by performing six replicate analyses of the samples through the complete analytical procedure from sample preparation to final result. The result revealed the precision with %RSD of system and method for CFX & PZD was found within the acceptable limit (≤ 2%), respectively. The results were shown in table 3(a) & 3(b).

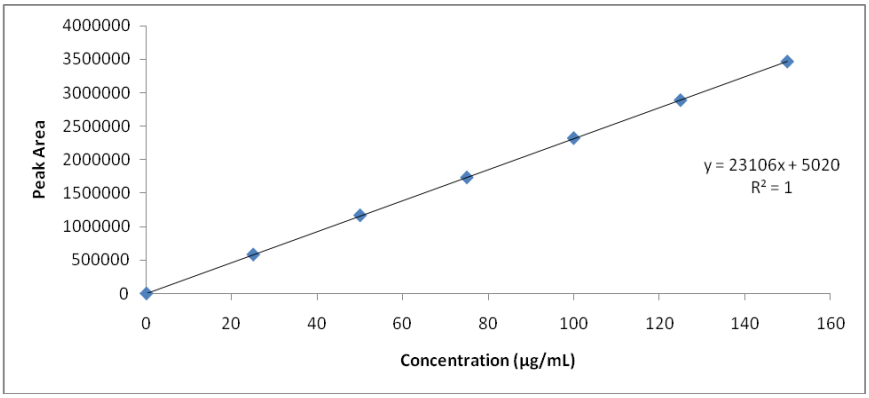

Fig. 2: Linear curve of CFX

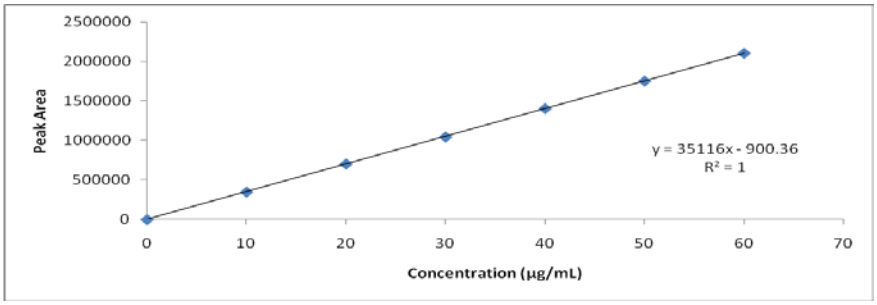

Fig. 3: Linear curve of PZD

Table 3(a): System precision of CFX and PZD

S. No.	CFX		PZD	
	RT	Area	RT	Area
1	2.770	2322825	4.106	1406389
2	2.776	2327209	4.084	1411186
3	2.778	2320825	4.087	1406761
4	2.789	2330949	4.116	1413490
5	2.778	2344886	4.089	1424263
6	2.778	2320852	4.087	1405880
Mean	2.778	2327924	4.095	1411328
Std Dev	0.006	9206.303	0.013	7028
% RSD	0.22	0.40	0.32	0.50

Table 3(b): Method precision of CFX and PZD

S. No.	CFX		PZD	
	RT	Area	RT	Area
1	2.772	2322835	4.102	1422882
2	2.775	2327405	4.085	1418542
3	2.777	2323325	4.082	1441521
4	2.785	2334512	4.108	1413524
5	2.765	2342541	4.084	1428541
6	2.782	2320541	4.085	1425412
Mean	2.776	2328527	4.091	1425070
SD	0.007	8446.39	0.011	9628.2
% RSD	0.26	0.36	0.27	0.68

Table 4: Intra-day and Inter-day precision

CFX	% Recovery			PZD	% Recovery		
	Day1	Day2	Day3[a]		Day1	Day2	Day3[a]
1	99.6	101.2	99.3	1	99.3	100.2	99.8
2	100.2	99.8	99.9	2	98.5	99.7	100.3
3	99.2	100.3	99.7	3	101.5	98.7	99.7
4	99.5	99.3	100.8	4	99.6	99.3	101.2
5	98.7	98.5	99.7	5	99.9	101.3	99.5
6	100.7	99.7	101.4	6	100.5	99.2	99.9
Intra-day (n=6)	99.65±0.71	99.8±0.91	100.1±0.79		99.8±1.03	99.73±0.92	100.06±0.61
Inter-day [b](n=18)		99.86±0.79				99.89±0.83	

[a]Different analyst, [b]mean±%RSD

Ruggedness (Intermediate precision)

The precision of the method was determined by intermediate precision studies. Intermediate precision was evaluated by comparing the assays on three different days using different analysts. The result revealed the precision with % RSD for intra-day and inter-day of CFX, was 0.91 & 0.79 and the precision with % RSD for intra-day and inter-day of PZD was 1.03 & 0.83, respectively. The results were shown in table 4.

Accuracy

To ensure the reliability and accuracy of the method, the recovery studies were carried out by adding a known quantity of the drug with pre-analysed sample and contents were re-analyzed by the proposed method. Accuracy was evaluated at three different concentrations equivalent to 75, 100, 125 % of the active ingredients, by adding a known amounts of CFX & PZD standard to a sample of known concentration and calculating the recovery of CFX & PZD with RSD (%) and % recovery for each concentration. Mean % recoveries of CFX & PZD were in between 99.29 to 100.57 & 99.36 to 100.02, respectively and were shown in table 5.

System suitability

To know reproducibility of the method system suitability test was employed to establish the parameters such as tailing factor, theoretical plates, resolution, asymmetry factor, and asymmetry (10 %), limit of detection and limit of quantification. The values were shown in table 6.

Table 5: Accuracy of the CFX & PZD

S. No.	75 % level		100 % level		125 % level	
	CFX	PZD	CFX	PZD	CFX	PZD
1	2321833	1411325	2875881	1754112	3467333	2086765
2	2322825	1402382	2865825	1756568	3425801	2104152
3	2311811	1408365	2868342	1754315	3441815	2113552
Avg	2318823	1407357	2870016	1754998	3444983	2101490
Recovered	75.43	75.01	99.29	99.60	124.34	124.20
Amt Present	75.00	75.00	100.00	100.00	125.00	125.00
% Recovery	100.57	100.02	99.29	99.60	99.47	99.36

Table 6: System suitability parameters

Parameter	Value	
	CFX	PZD
Retention time (min)	2.783	4.111
Theoretical plates	4265	7273
Tailing Factor	1.27	1.33
Resolution	-	7.36
Symmetry Factor	1.12	1.08
% RSD of peak area (n=6)	0.40	0.50
% RSD of retention time	0.40	0.50

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately changed. To study the effect of eluent flow rate (Changed from 1.0 to 1.2 ml/min), mobile phase ratio [Changed from 60:40 to 65:35 (v/v) and to 55:45 (v/v)]. In all the above varied conditions, the proposed method indicating that the test method was robust for all variable conditions. Hence the method was sufficiently robust for normally expected variations in chromatographic conditions. The results were shown in table 7.

Table 7: Robustness of the method

Condition	Variation	Mean area*±% RSD		Mean (R_t)*±% RSD	
		CFX	PZD	CFX	PZD
Mobile phase composition	55: 45	2101003±0.24	1314639±0.56	2.766±0.52	3.939±0.37
	50:50	2322841±0.45	1426389±0.12	2.783±0.24	4.111±0.29
	45:55	2095095±0.72	1267540±0.74	2.753±0.77	3.717±0.34
Mobile phase flow rate (ml/min)	0.8	2128803±0.49	1293180±0.72	2.530±0.21	3.723±0.17
	1	2327222±0.56	1411145±0.73	2.776±0.48	4.084±0.82
	1.2	2595235±0.73	1583206±0.17	3.07±0.64	4.532±0.13

*Mean of six values

Table 8: LOD & LOQ

Formula		CFX		PZD	
		Mean*±SD		Mean*±SD	
LOD	LOQ	LOD (µg/ml)	LOQ (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)
3.3 σ/S	10 σ/S	1.44±0.04	4.38±0.07	0.964±0.03	2.92±0.05

*Mean of six values

Selectivity

Results of stress testing studies indicated a high degree of selectivity of this method for CFX & PZD. The degradation of CFX & PZD was found to be similar for both the tablets and API powder.

LOD and LOQ

Limits of Detection (LOD) and Quantification (LOQ), the limits of detection and quantification were calculated by the method based on the standard deviation (σ) and the slope (S) of the calibration plot, using the formulae LOD = 3.3 σ/S and LOQ =10 σ/S. The results were shown in table 8.

Assay of the method

The assay of commercial tablets was established with present chromatographic condition developed and it was found to be more accurate and reliable. The average drug content was found to be 100.15 & 99.53 of the labelled claim of CFX & PZD, respectively. No interference peaks were found in chromatogram, indicating that the estimation of drug free from interference of excipients. The results were shown in the table-9. The chromatogram of bulk and marketed formulation was shown in fig.4 and fig. 5.

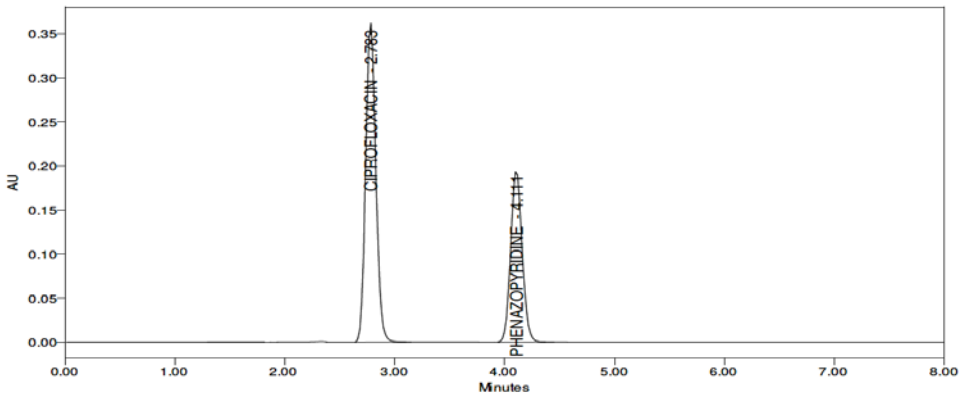

Fig. 4: A typical chromatogram of CFX with PZD in bulk drug mixture

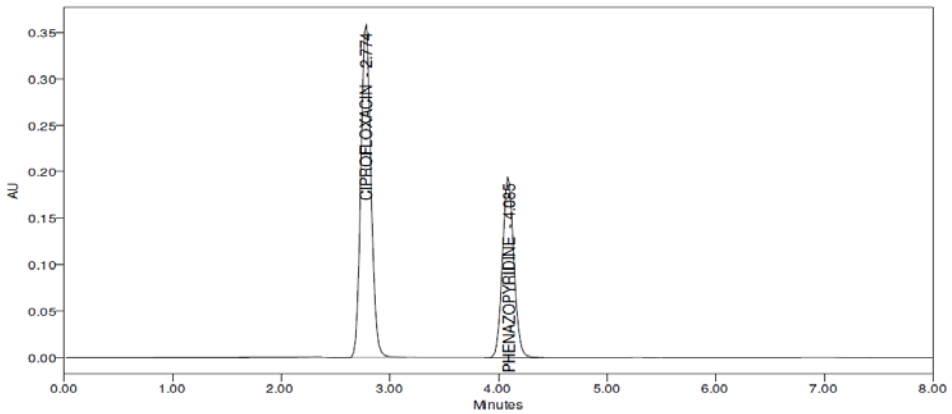

Fig. 5: A typical chromatogram of CFX with PZD in dosage form

Table 9: Assay of the method

Drug	Label claim (mg)	Drug content (%)*	% RSD
CFX	250	100.15	0.27
PZD	100	99.53	0.49

*Mean of three values

CONCLUSION

An isocratic RP-HPLC method developed for simultaneous determination of CFX and PZD in bulk and tablet dosage form. The validation data demonstrate good precision and accuracy, which prove the reliability of the proposed method. The short runtime and simple extraction procedure is advantageous for analyzing routine quality control sample of CFX and PZD.

CONFLICT OF INTERESTS

Declared None

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