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METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF CEFIXIME AND OFLOXACIN IN A PHARMACEUTICAL FORMULATION BY RP-HPLC METHOD

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ABSTRACT

An isocratic Simultaneous estimation by RP-HPLC Method were developed and validated for the quantification of Cefixime and Ofloxacin in tablet dosage form. Quantification was achieved by using a reversed-phase C18 column (INERTSIL Column, 5µ, 250 mm × 4.6 mm) at ambient temperature with mobile phase consisting of Ammonium acetate Buffer buffer: Acetonitrile: Methanol (50:30:20 pH:6.5)). The flow rate was 1.0 ml/min. Measurements were made at a wavelength of 226nm. The average retention time were found to be 2.39 min for Cefixime and 4.06 min for Ofloxacin. The proposed method was validated for selectivity, precision, linearity and accuracy. The assay methods were found to be linear from 60-140µg/ml for Cefixime and 60-140µg/ml for Ofloxacin. All validation parameters were within the acceptable range. The developed method was successfully applied to estimate the amount of Ofloxacin and Cefexime in tablet dosage form.

KEYWORDS

Cefixime, Ofloxacin, RP-HPLC method, Inertsil ODS Column, Different solvent such as Methanol, Acetonitrile, Ammonium acetate, Ortho phosphoric acid and Validation.

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INTRODUCTION

Ofloxacin (Figure No.1 (a)) a synthetic fluoroquinolone (fluoroquinolones) antibacterial agent that inhibits the super coiling activity of bacterial DNA gyrase, halting DNA replication. Ofloxacin acts on DNA gyrase and toposiomerase IV, enzymes which, like human topoisomerase, prevents the excessive super coiling of DNA during replication or transcription. By inhibiting their function, the drug thereby inhibits normal cell

division. Elimination is mainly by renal excretion. Between 65% and 80% of an administered oral dose of Ofloxacin is excreted unchanged via the kidneys within 48 hours of dosing. Four to eight percent of an Ofloxacin dose is excreted in the feces. This indicates a small degree of biliary excretion of Ofloxacin. Side Effects: Headache, dizziness, dry mouth, nervousness and flushing[1].

Cefixime (Figure No.1 (b)) is an antibiotic, is a third-generation cephalosporin like ceftriaxone and cefotaxime. Cefixime is highly stable in the presence of beta-lactamase enzymes. As a result, many organisms resistant to penicillins and some cephalosporins due to the presence of beta-lactamases, may be susceptible to cefixime. The antibacterial effect of cefixime results from inhibition of mucopeptide synthesis in the bacterial cell wall. Like all beta-lactam antibiotics, cefixime binds to specific penicillin-binding proteins (PBPs) located inside the bacterial cell wall, causing the inhibition of the third and last stage of bacterial cell wall synthesis. Cell lysis is then mediated by bacterial cell wall autolytic enzymes such as autolysins; it is possible that cefixime interferes with an autolysin inhibitor. Side Effects: drowsiness, sweating, dry mouth, headache, skin problems, lethargy, gastrointestinal irritation, hypersensitivity reactions, as well as movement problems/muscle rigidity and tremor[2].

MATERIALS AND METHOD

Instruments the chromatographic technique performed on a Shimadzu LC20-AT Liquid chromatography with SPD-20A prominence UV-visible detector and Spinchrom software, reversed phase C18 column (Inertsil 5µ, 250 mm × 4.6 mm) as stationary phase. Thermo Electron Corporation double beam UV-visible spectrophotometer (vision pro-software), Ultrasonic cleaner, Shimadzu analytical balance AY-220, Vaccum micro filtration unit with 0.45µ membrane filter was used in the study.

MATERIALS

Pharmaceutically pure sample of Cefixime and Ofloxacin bulk drugs were obtained as gift samples from Chandra laboratories Pvt Ltd, Prashanthi nagar, Kukatpally, Hyderabad, India. The purity of the drug was evaluated by obtaining its melting point and ultraviolet (UV) and infrared (IR) spectra. No impurities were found. The drug was used without further purification. HPLC-grade Acetonitrile and Methanol ware from standard reagents Pvt Ltd. Ammonium acetate (AR grade) was from Merck. A tablet formulation of Cefixime and Ofloxacin bulk drugs (200 mg and 200mg label claims) was procured from local market (Miliximе-O, Glenmark, India).

Determination of Working Wavelength (λmax)

10 mg of the Ofloxacin standard drug is taken in a 10 ml volumetric flask and dissolved in methanol and volume made up to the mark, from this solution 0.1ml is pipetted into 10 ml volumetric flask and made upto the mark with the methanol to give a concentration of 10 µg/ml. The above prepared solution is scanned in uv between 200-400 nm using methanol as blank. The λmax was found to be 281nm.

10 mg of the Cefixime standard drug is taken in a 10 ml volumetric flask and dissolved in methanol and volume made up to the mark, from this solution 0.1ml is pipetted into 10 ml volumetric flask and made upto the mark with the methanol to give a concentration of 10 µg/ml. The above prepared solution is scanned in UV between 200-400 nm using methanol as blank. The λmax was found to be 216nm. The iso bestic point of Ofloxacin and Cefixime were found to be 226nm (Figure No.2).

Preparation of mobile phase[2-9]

Buffer Preparation

3.85gm of Ammonium acetate was weighed and dissolved in 100ml of water and volume was made up to 1000ml with water. Adjust the pH to 6 .5 using triethylamine. The buffer was filtered through 0.45µ filters to remove all fine particles and gases.

Mobile phase

A mixture of 50 volumes of Ammonium acetate Buffer, 30 volumes of methanol and 20 volumes of Acetonitrile (HPLC grade). The mobile phase was sonicated for 10min to remove gases.

Analysis of formulation

Preparation of standard solution

A 100mg of standard Ofloxacin and 100 mg Cefixime ware weighed and transferred to 50 ml of volumetric flask and dissolved in mobile phase. The flask was shaken and volume was made up to mark with mobile phase to give a primary stock solution containing 1000µg/ml Ofloxacin and and 1000µg/ml of Cefixime. From the above solution 5ml of solution is pipetted out into a 50 ml volumetric flask and volume was made up to mark with mobile phase to give a solution containing 100µg/ml Ofloxacin and 100µg/ml of Cefixime.

Preparation of sample solution

For the estimation of the drug in tablet formulation twenty tablets were weighed and their average weight was determined. The tablets were then finely powdered. Appropriate quantity equivalent to 100mg Ofloxacin and 100 mg Cefixime ware accurately weighed and The powder was transferred to 100 ml volumetric flask and shaken vigorously with mobile phase and sonicated for 15 min and volume made up to the mark with mobile phase. The solution was shaken vigorously and filtered by using whatmann filter no.41. from the above filtered clear solution 5ml of sample pipetted out into a 50 ml volumetric flask volume made up to the mark with mobile phase to give a solution containing 100µg/ml Ofloxacin and 100µg/ml of Cefixime. Calculation 5 replicates of each of sample and standard solutions were injected and their average peak areas were taken.

The amount of Ofloxacin and Cefixime present in the formulation by using the formula given below:-

$$\% \text{ Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{AW}{LC} \times 100$$

Where,

AS: Average peak area due to standard preparation

AT: Peak area due to assay preparation

WS: Weight of standard drug taken

WT: Weight of sample in assay preparation

DT: Dilution of assay preparation

DS: Dilution of standard preparation

AW: Average weight of 20 tablets

LC: Label claim

P: Purity of standard drug.

METHOD VALIDATION

Linearity

Linearity was studied by analyzing five standard solutions covering the range of 60-140µg/ml for Ofloxacin and 60-140 µg/ml for Cefixime of the drug. From the primary stock solution 0.6ml, 0.8ml, 1.0ml, 1.2ml, 1.4 ml of aliquots are pipetted into 10 ml volumetric flasks and made up to the mark with the mobile phase to give a concentrations of 60µg/mL, 80µg/mL, 100 µg/mL, 120µg/mL and 140µg/mL of Ofloxacin and 60µg/mL, 80µg/mL, 100 µg/mL, 120µg/mL and 140µg/mL mL of Cefixime (Table No.1 and 1.1).

Calibration curve (Figure No.3.1 and 3.2) with concentration verses peak areas was plotted by injecting the above prepared solutions and the obtained data were subjected to regression analysis using the least squares method.

Method precision (repeatability)

The precision of the instrument was checked by repeated injections and measurement of peak areas and retention times of solutions (n = 6) for, 100 µg/ml of Ofloxacin and 100 µg/ml of Cefixime without changing the parameter of the proposed chromatographic method.

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were separately determined based on standard deviation of the y-intercept and the slope of the calibration curve by using the equations (2) and (3), respectively (Table No.2).

$$\textbf{LOD} = 3.3\,\delta/S \quad \textbf{……. (3)}$$

$$\textbf{LOQ} = 10\,\delta/S \quad \textbf{…….. (4)}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

Accuracy (recovery study)

The accuracy of the method was determined by calculating the recoveries of Ofloxacin and Cefixime by the standard addition method. Known amounts of

standard solutions of Ofloxacin and Cefixime were added at 20% concentration to pre quantified sample solutions of Ofloxacin (100, 120, 140µg/ml) and Cefixime (100, 120, 140µg/ml). The amount of Ofloxacin and Cefixime recovered was estimated by using the following formulas (Table No.3 (a and b).

$$\% \text{ Recovery} = \frac{\text{amount found}}{\text{Amount added}} \times 100$$

$$\text{Amount Found}(mcg/ml) = \frac{\text{Mean test area}}{\text{Mean standard area}} \times \text{Standard concentration}$$

Specificity

In an assay, demonstration of specificity requires that it can be shown that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients and demonstrating that the assay results are unaffected by the presence of these extraneous materials. There should be no interference of the diluents, placebo at retention time of drug substances (Figure No.4).

Robustness

Robustness is the measure of a method remain unaffected by small, deliberate changes in method parameters like flow rate and detection wavelength on assay of the analyte of interest. Here the detection wavelength varied ±2nm and flow rate was varied ±0.2 ml/min. The results were shown in (Table No.4).

Ruggedness

The ruggedness of the method was studied by analyzing the sample and standard preparations by two analysts. The % RSD assay values between two analysts was calculated i.e., (limit <2%).

This indicates the method was rugged. The results were shown in Table No.5.

RESULTS AND DISCUSSION

In RP HPLC method, the primary requirement for developing a method for analysis is that the using different solvents and buffers and columns to get better retention time and theoretical plates, and better cost effective and time saving method than the previously developed methods. The iso bestic point of Ofloxacin and Cefixime were found to be 226nm (Figure No.2) by scanning in UV region. The chromatographic method was optimized with mobile phase consisting of Ammonium acetate: Acetonitrile: Methanol (50:20:30) and C18 Inertsil column. All the validation parameters were studied at a wavelength 226nm. Accuracy was determined by calculating the recovery (Table No.3) and the results were in acceptable range (limit 98-102%). The method was successfully used to determine the amount of Ofloxacin and Cefixime present in the Tablet. The results obtained were in good agreement with the corresponding labeled amount (Table No.3). The method was linear in the concentration range of 60 to 140µg/ml for Ofloxacin and 60 to 140µg/ml for Cefixime (Table No.1 and 1.1 and Figure No.5). Robustness and ruggedness results were in acceptable range (Table No.4 and Table No.5). The assay was performed for both drug and the results showed in Table No.6 and Figure No.6 and 7). Precision was calculated as repeatability and intra and inter day variations (% RSD) for the drug (Table No.7 and 8). Summary of all validation parameters for method is given in Table No.9. By observing the validation parameters, the method was found to be simple, sensitive, accurate and precise. Hence the method can be employed for the routine analysis Ofloxacin and Cefixime in tablet dosage form.

Table No.1: Linearity of Ofloxacin

S.No	Concentration (µg/ml)	Peak Area
1	60	477.514
2	80	627.073
3	100	727.216
4	120	868.97
5	140	1018.025

Table No.1.1: Linearity of Cefexime

S.No	Concentration (µg/ml)	Peak Area
1	60	537.745
2	80	705.467
3	100	840.679
4	120	979.036
5	140	1158.544

Table No.2: LOD and LOQ values from calibration curve

S.No	Cefixime		Ofloxacin	
	Concentration µg/ml	Peak Area	Concentration µg/ml	Peak Area
1	60	477.514	60	537.745
2	80	627.073	80	705.467
3	100	727.216	100	840.679
4	120	868.97	120	971.036
5	140	1018.025	140	1158.544
S.D	31.6	210	31.623	239
Slope	6.6		7.535	

Table No.3(a): Recovery data for Cefixime

S.No	Recovery level	Accuracy Cefixime					Average % Recovery
		Amount taken(mcg/ml)	Area	Average area	Amount recovered (mcg/ml)	% Recovery	
1	80%	100	780.779	776.221	99.03	99.03	100.05%
		100	767.105				
		100	780.779				
2	100%	120	874.332	876.445	120.52	100.43	
		120	877.992				
		120	877.012				
3	120%	140	1029.217	1020.963	140.99	100.71	
		140	1018.025				
		140	1015.646				

Table No.3(b): Recovery data for Ofloxacin

S.No	Recovery level	Accuracy Ofloxacin					Average % Recovery
		Amount taken (mcg/ml)	Area	Average area	Amount recovered (mcg/ml)	% Recovery	
1	80%	100	876.196	873.127	99.01	99.01	99.58%
		100	866.989				
		100	876.196				
2	100%	120	987.754	988.846	117.62	98.02	
		120	986.68				
		120	992.103				
3	120%	140	1173.701	1161.750	142.40	101.71	
		140	1158.544				
		140	1153.004				

Table No.4: Results of Robustness study

S.No	Parameter	Cefixime		Ofloxacin	
		Retention time (min)	Tailing factor	Retention time(min)	Tailing factor
1	**Flow Rate**				
	0.8 ml/min	2.983	1.704	5.080	1.400
	1.0 ml/min	2.410	1.783	4.083	1.594
	1.2 ml/min	2.043	1.650	3.450	1.464
2	**Wavelength**				
	224nm	2.407	1.696	3.590	1.424
	226nm	2.410	1.783	4.083	1.594
	228nm	2.390	1.652	4.087	1.382

Table No.5: Results of Ruggedness

S.No	Cefixime	% Assay	Ofloxacin	% Assay
1	Analyst 01	100.07	Analyst 01	99.50
2	Anaylst 02	100.2	Anaylst 02	99.70
3	% RSD	0.09%	% RSD	0.141%

Table No.6: Assay Results

S.No	Cefixime			Ofloxacin	
	Injections	**Standard Area**	**Sample Area**	**Standard Area**	**Sample Area**
1	Injection-1	766.15	767.951	870.067	866.991
2	Injection-2	767.386	769.224	873.384	866.11
3	Injection-3	770.258	770.067	870.812	869.701
4	Injection-4	767.029	770.197	876.733	860.68
5	Injection-5	769.113	766.992	863.079	876.514
6	Average Area	767.987	768.886	870.815	867.9992
Tablet average weight		720.1mg		720.1mg	
Standard weight		50 mg		50 mg	
Sample weight		180.2mg		180.2mg	
Label amount		200 mg		200 mg	
Std.purity		99.6		99.8	
Amount found in mg		199.24 mg		198.76 mg	
Assay (% Purity)		99.62 %		99.38 %	

Table No.7: Method Precision (Repeatability)

S.No	Cefexime		Ofloxacin	
	Rt	**Area**	**Rt**	**Area**
1	2.427	777.216	4.110	866.679
2	2.393	770.533	4.067	873.18
3	2.383	763.404	4.057	863.577
4	2.410	763.692	4.083	859.668
5	2.403	762.503	4.080	863.186
6	2.383	764.619	4.057	866.021
Avg	2.3998	766.995	4.076	865.385
St.dev	0.0171	5.773	0.020	4.553
% RSD	0.71	0.75	0.49	0.53

Table No.8: Intraday Precision

S.No	Cefexime		Ofloxacin	
	Rt	Area	Rt	Area
1	2.400	769.362	4.025	866.254
2	2.401	765.565	4.085	867.352
3	2.403	763.254	4.025	865.988
4	2.401	769.328	4.096	864.285
5	2.391	766.222	4.098	863.985
6	2.396	768.521	4.021	865.321
avg	2.3987	767.0420	4.0583	865.5308
stdev	0.0044121	2.449429	0.038261	1.267624
% RSD	0.18393988	0.319334	0.942764	0.146456

Interday Precision

S.No	Cefexime		Ofloxacin	
	Rt	Area	Rt	Area
1	2.458	769.854	4.025	864.251
2	2.453	769.325	4.036	863.241
3	2.451	766.501	4.021	867.212
4	2.469	767.451	4.085	866.325
5	2.478	769.52	4.087	866.854
6	2.495	764.458	4.091	867.542
avg	2.4673	767.8515	4.0575	865.9042
stdev	0.01697842	2.119679	0.033465	1.74915
% RSD	0.68812825	0.276053	0.824767	0.202003

Table No.9: Validation parameters of evaluated method

S.No	Parameter	Limit	Value Obtained
1	Accuracy (% Recovery)	98-102%	99.58 % (Ofloxacin) 100.05% (Cefexime)
2	Linearity concentrations Range (µg/mL) Regression coefficient (R2 value)	NLT 0.99%	60 to 140 µg/ml (Ofloxacin) R^2=0.996 and 60 to 140 µg/ml (Cefexime) R^2=0.9962
3	Precision (% RSD) Method precision(Repeatability) (%RSD, n = 6)	NMT 1%(For Rt) NMT 2%(For Area)	%RSD of Rt=0.71% and %RSD of Area 0.75% (Ofloxacin) %RSD of Rt=0.49% and %RSD of Area 0.53% (Cefexime)
4	Intermediate Precision	-	-
5	Robustness(%assay)	It should be meet system suitability Parameters	Met the acceptance criteria
6	Ruggedness (% RSD analyst to analyst variation)	NMT 2%	% RSD of Ofloxacin:0.09% % RSD of Ofloxacin:0.141%

aSD=Standard deviation, bLOD = Limit of detection, cLOQ = Limit of quantification, dRSD = Relative standard deviation.

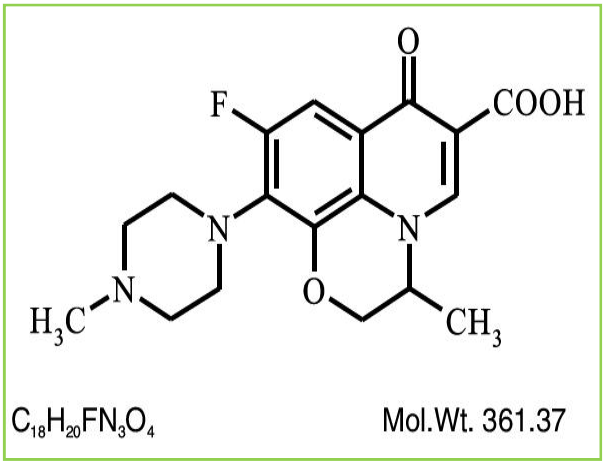


Figure No.1 (a): Structure of Ofloxacin

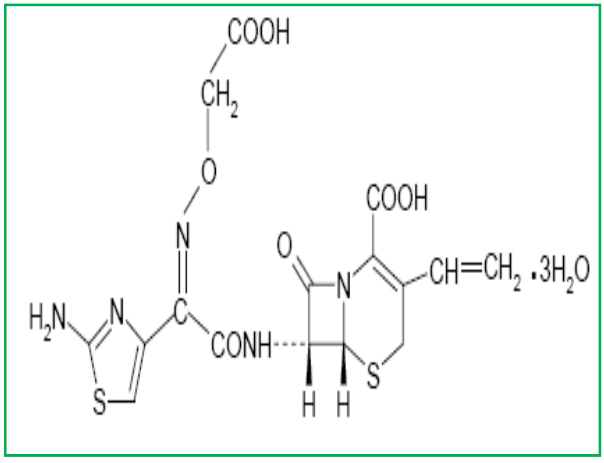


Figure No.1 (b): Structure of Cefexime

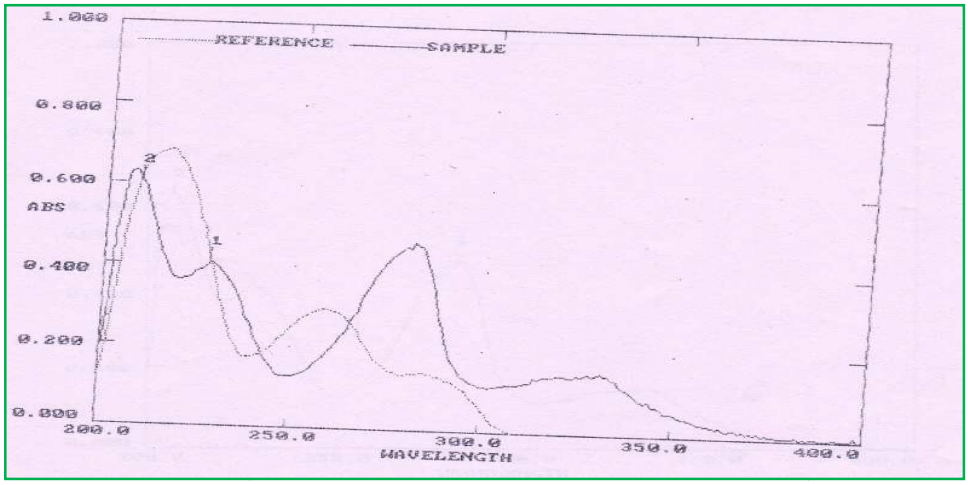


Figure No.2: Determination of Isobestic Point

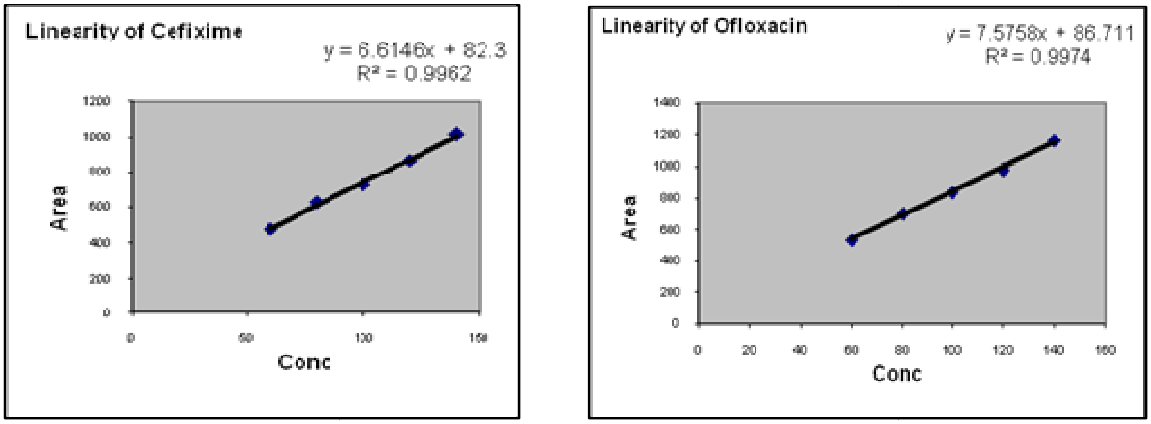


Figure No.3.1 and 3.2: Linearity (calibration) curve of Ofloxacin and Cefexime

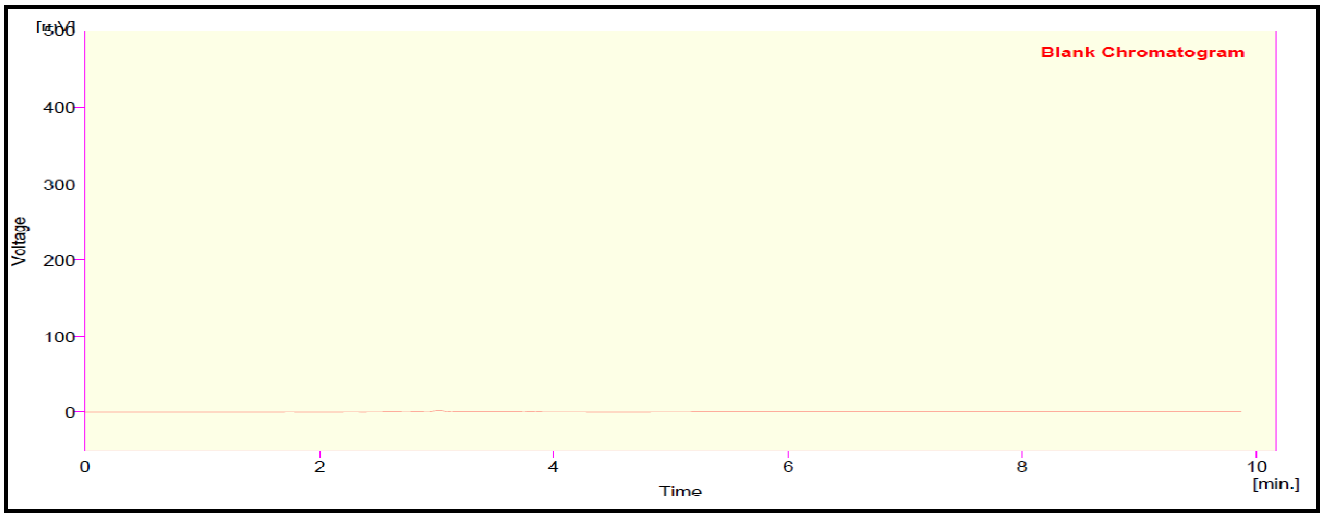
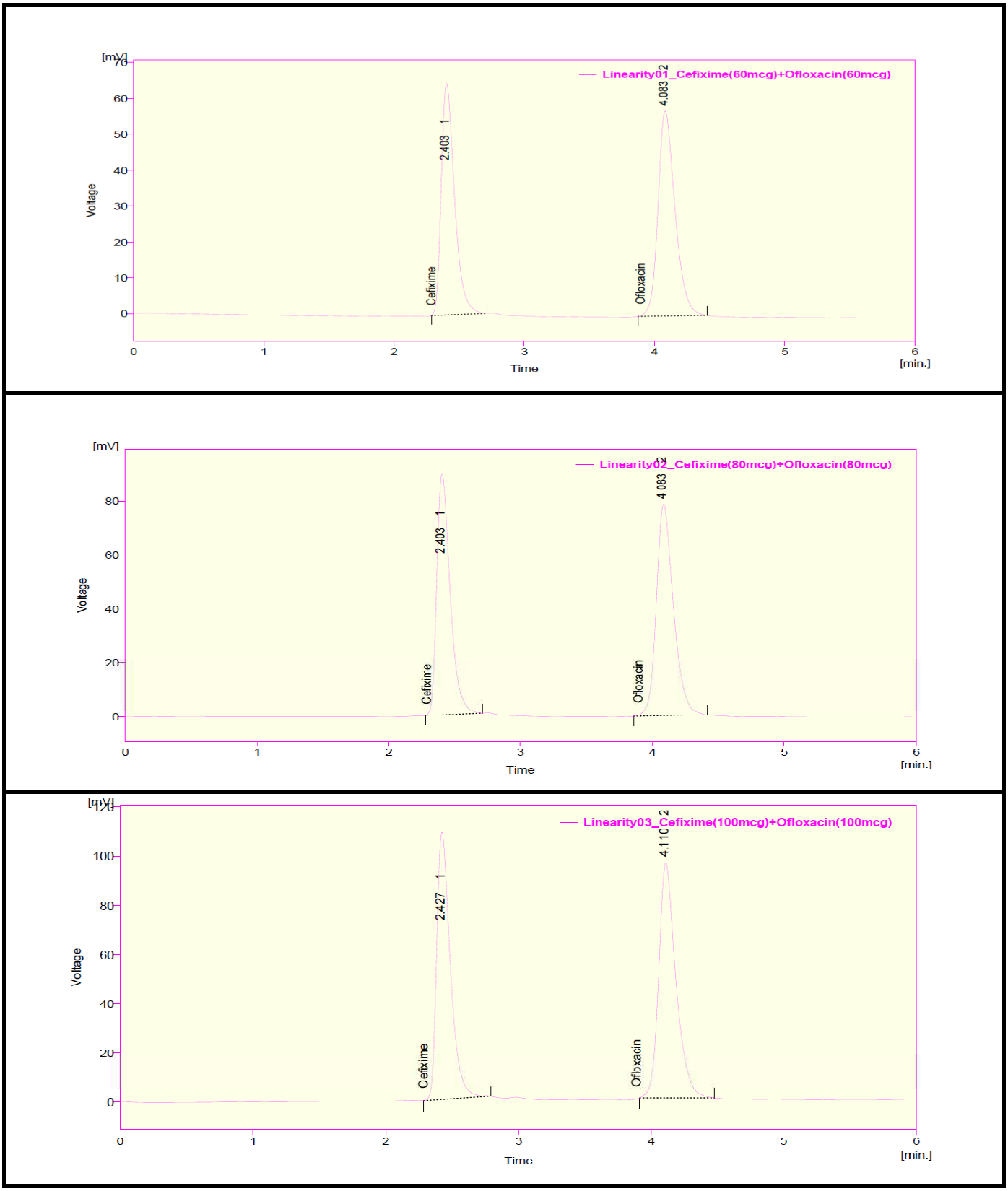


Figure No.4: Chromatograms of Specificity (placebo, blank preparations)


[mV]
Linearity01_Cefixime(60mcg)+Ofloxacin(60mcg)
2.403
1
Cefixime
4.083
2
Ofloxacin
Voltage
Time
[min.]
[mV]
Linearity02_Cefixime(80mcg)+Ofloxacin(80mcg)
2.403
1
Cefixime
4.083
2
Oficxacin
Voltage
Time
[min.]
[mV]
Linearity03_Cefixime(100mcg)+Ofloxacin(100mcg)
2.427
1
Cefixime
4.110
2
Ofbxacin
Voltage
Time
[min.]

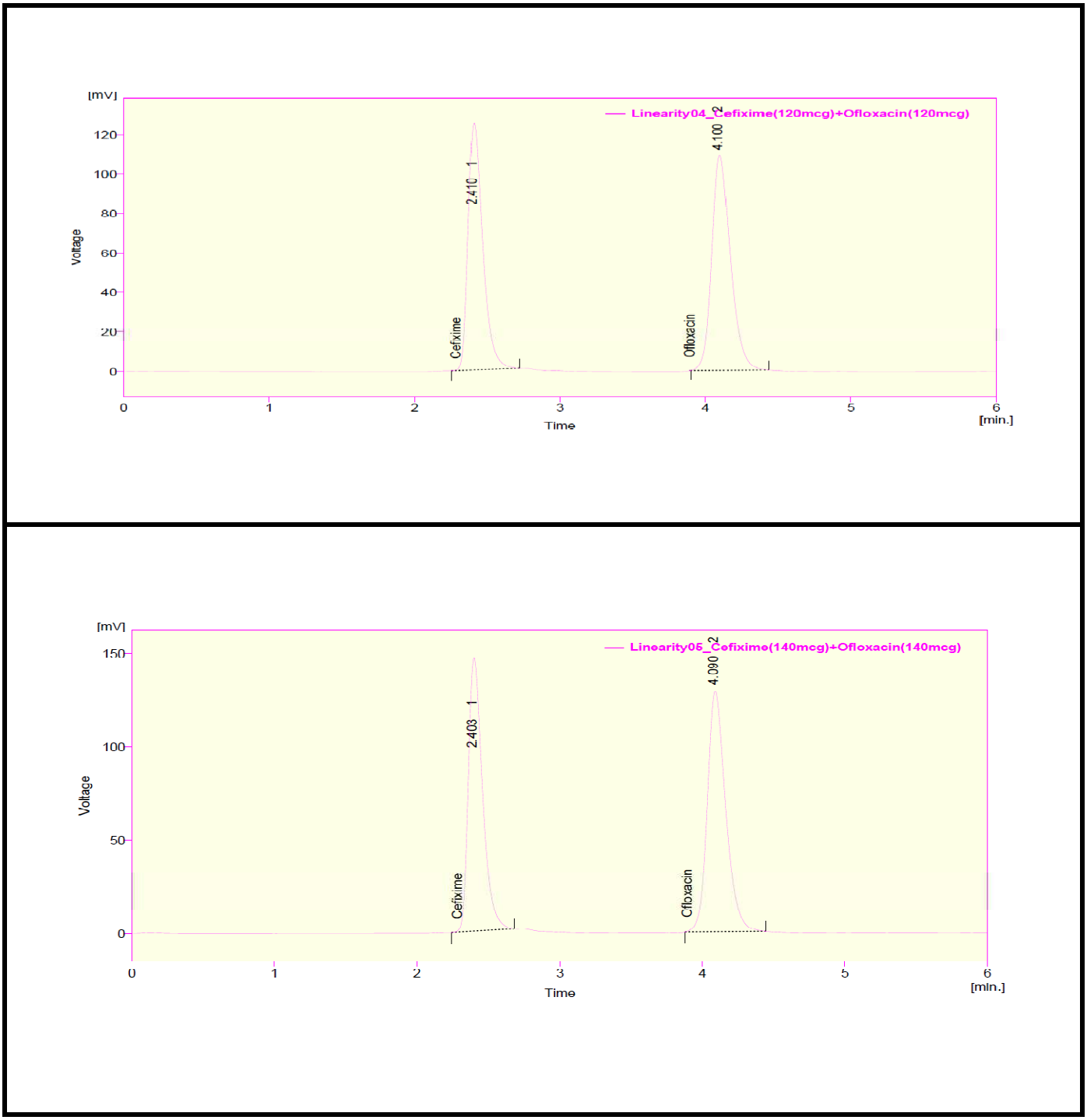


Figure No.5: Chromatograms of Linearity

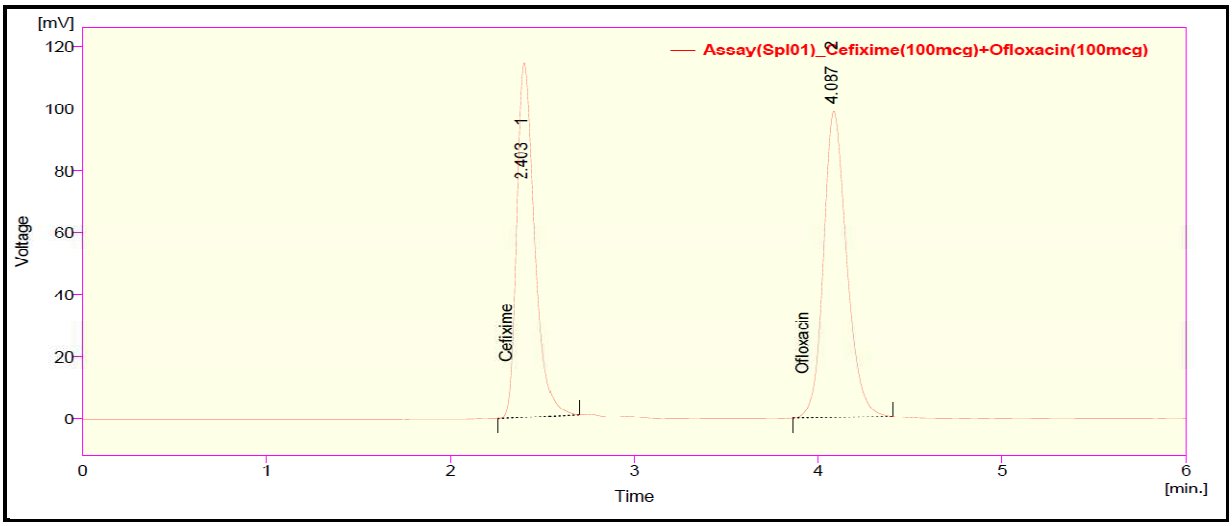


Figure No.6: Chromatogram of Assay sample preparation

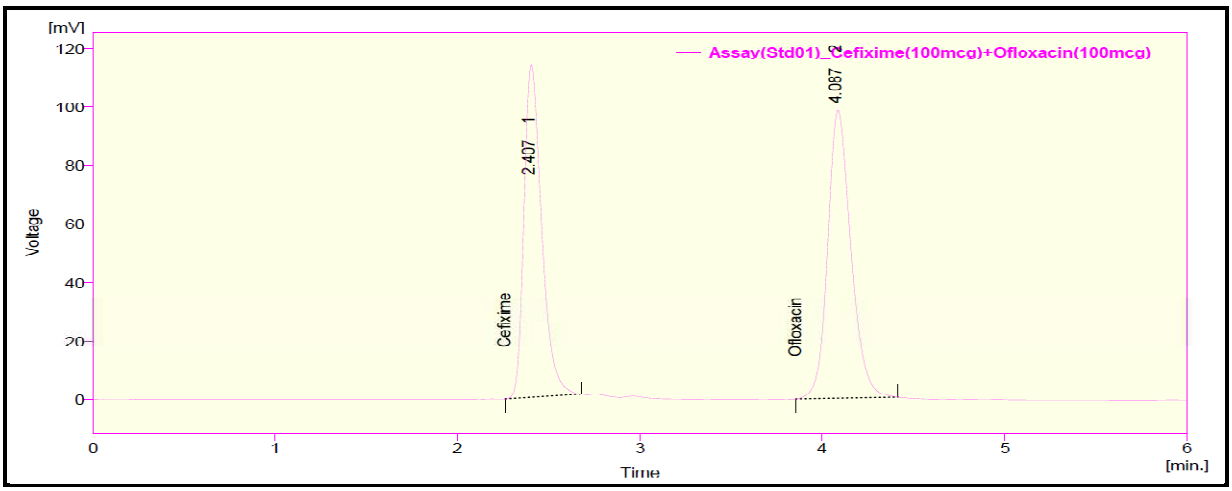


Figure No.7: Chromatogram of assay standard preparation

CONCLUSION

The proposed Simultaneous Estimation by RP-HPLC method was found to be simple, sensitive, accurate and precise for determination of Ofloxacin and Cefexime in tablet. The method utilizes easily available and cheap solvent for analysis of Ofloxacin and Cefexime hence the method was also economic for estimation of Ofloxacin and Cefexime from Tablet. The common excipients and other additives are usually present in the Tablet mixture does not interfere in the analysis of Ofloxacin and Cefexime; hence it can be conveniently adopted for routine quality control analysis of the drug in pharmaceutical formulation.

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