Development and validation of new RP-HPLC method for simultaneous estimation of drug Cefixime and Azithromycin in tablet dosage form

Kadari Nagaraju
K.C Reddy Institute of Pharmaceutical Sciences, Medikonduru, Guntur (Dt), A.P
***Corresponding author: E-Mail: anjipeddinti2245@gmail.com**

ABSTRACT

A simple, economic, accurate and precise reverse phase high performance liquid chromatography method for analysis of Cefixime and Azithromycin was developed and validated according to ICH guidelines. The quantification of the drug was carried out using PDA (photodiode array) detector. Column in isocratic mode, with mobile phase Methanol:Buffer in ratio of 85:15 was used. The flow rate was 1.0 ml/min and effluent was monitored at 275 nm.the retention times were 2.844 and 3.538min for Cefixime and Azithromycin respectively. The injection volume was 20µl.as per ICH guidelines the method was validated and the method was found to be linear in the range of 20-80µg/ml for Cefixime and Azithromycin. Percent recovery studies of Cefixime and Azithromycin 98.00%and101.50%. The limit of detection and quantification was found to be 0.34&1.05µg/ml Cefixime and 0.25&0.34µg/ml for Azithromycin .the values of precession and robustness lie within the acceptance limit. Thus the proposed method can be successfully applied for simultaneous determination of Cefixime and Azithromycin in routine analysis work.

Key Words: Cefixime, Azithromycin, liquid chromatography, RP-HPLC, Validation

1. INTRODUCTION

Cefixime, 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.

Azithromycin is a semi-synthetic macrolide antibiotic of the azalide class. Like other macrolide antibiotics, azithromycin inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit of the bacterial 70S ribosome. Binding inhibits peptidyl transferase activity and interferes with amino acid translocation during the process of translation. Its effects may be bacteriostatic or bactericidal depending of the organism and the drug concentration. Its long half-life, which enables once daily dosing and shorter administration durations, is a property distinct from other macrolides.

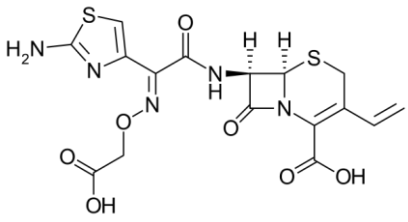

Figure.1.Cefixime structure

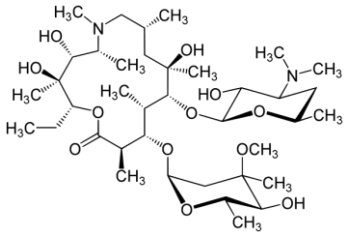

Figure.2.Azithromycin structure

2. MATERIALS AND METHODS

RP-HPLC method for the simultaneous estimation of Cefixime and Azithromycin. Instruments used are HPLC:Waters-2690 alliance separation Module (isocratic system), Column: Hypersil C_{18} column (Length 250 mm, diameter 4.6mm, particle size:5µm), Detector:Waters-996 photodiode array detector, Data handling system(Waters empower software).

Reagents and chemicals used are Methanol (HPLC grade) Standard reagents private limited Hyderabad, Acetonitrile (HPLC grade) Standard reagents private limited Hyderabad, Water (HPLC grade) Standard reagents private limited Hyderabad, Buffer(KH2PO4)Hplc Grade.

Drug sample**:** Cefixime and Azithromycin raw material

Method development for HPLC: Degassed Methanol and Potassium Dihydrogen Phosphate Buffer in the ratio of 55:45 V/V.

Preparation of (KH_2PO_4 0.1M) buffer: Weight 3.8954g of di-sodium hydrogen phosphate and 3.4023 of potassium dihydrogen phosphate in to a beaker containing 1000ml of distilled water and dissolve completely. Then ph is adjusted with orthophosphoric acid and then filtered through 0.45µm membrane filter.

Preparation of stock solution: Reference solution: The solution was prepared by dissolving 20.0 mg of accurately weighed Cefixime and 25.0 mg Azithromycin in Mobile phase, in two 100.0 mL volumetric flasks separately and sonicate for 20min. From the above solutions take 10.0 mL from each solution into a 50.0 mL volumetric flask and then makeup with mobile phase and sonicate for 10min.

Preparation of working standard solution: The stock solutions equivalent to 20ppm to 80ppm with respect to both drugs were prepared in combination of Cefixime and Azithromycin above, sonicated and filtered through 0.45µ membrane.

Table.1.Optimized chromatographic conditions

Parameters	Method
Stationary phase (column)	Inertsil -ODS C_{18}(250 x 4.6 mm, 5 µ)
Mobile Phase	Methanol : Buffer (85:15)
Flow rate (ml/min)	1.0 ml/min
Run time (minutes)	10 min
Column temperature (°C)	Ambient
Volume of injection loop (µl)	20
Detection wavelength (nm)	275nm
Drug RT (min)	2.186min for Cefixime and 3.968 for Azithromycin

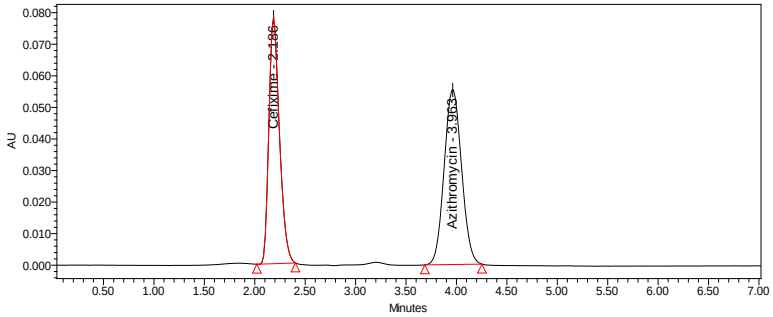

Figure.3.Chromatogram of optimized method

Method validation: An integral part of analytical method development is validation. Once the method has been devised, it is necessary to evaluate under the conditions expected for real samples before being used for a specific purpose. The following parameters were evaluated.

System suitability: A Standard solution was prepared by using Cefixime and Azithromycin working standards as per test method and was injected Five times into the HPLC system.

The system suitability parameters were evaluated from standard chromatograms by calculating the % RSD from five replicate injections for Cefixime and Azithromycin retention times and peak areas.

Acceptance criteria:

1. The % RSD for the retention times of principal peak from 5 replicate injections of each Standard solution should be not more than 2.0 %
2. The % RSD for the peak area responses of principal peak from 5 replicate injections of each standard Solution should be not more than 2.0%.
3. The number of theoretical plates (N) for the Cefixime and Azithromycin peaks is NLT 3000.
4. The Tailing factor (T) for the Cefixime and Azithromycin peaks is NMT 2.0

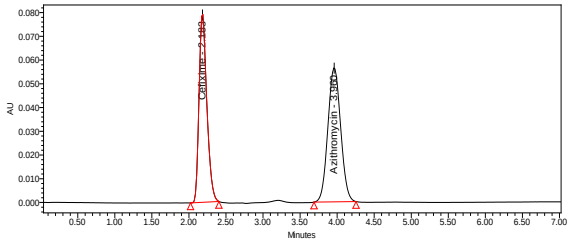

Figure.4.System suitability Chromatogram for standard – 1

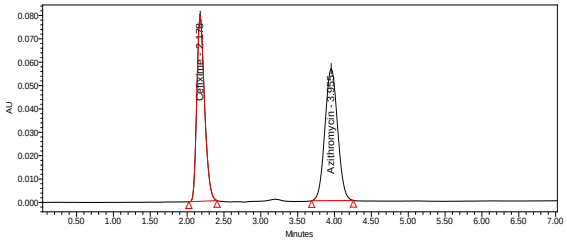

Figure.5.System suitability Chromatogram for standard – 2

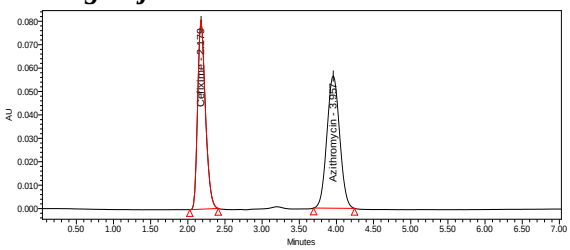

Figure.6.System suitability Chromatogram for standard – 3

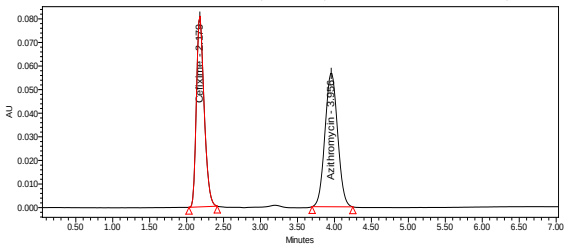

Figure.7.System suitability Chromatogram for standard - 4

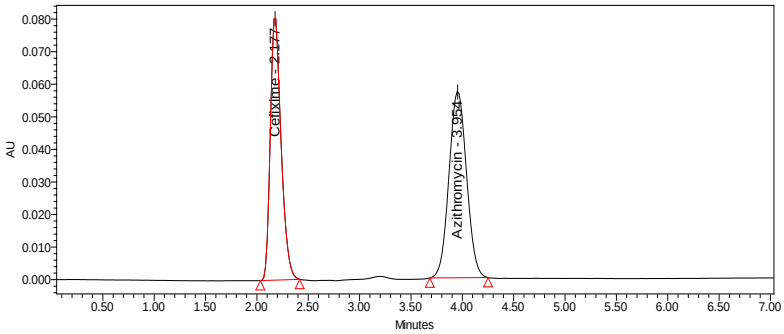

Figure.8.System suitability Chromatogram for standard – 5

Observation: The %RSD for retention times and peak areas were found to be within the limit. Refer table: 1 As shown in fig 4 – 8.

Specificity:

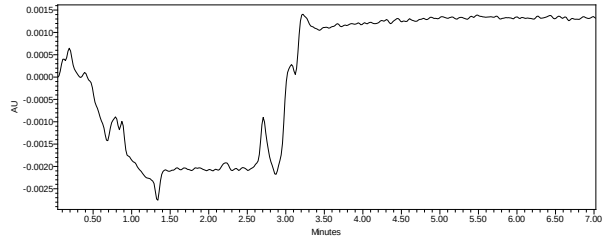

Figure.9.Chromatogram of standard

Cefixime and Azithromycin: Solutions of standard and sample were prepared as per the test method are injected into chromatographic system.

Acceptance criteria: Chromatograms of standard and sample should be identical with near Retention time.

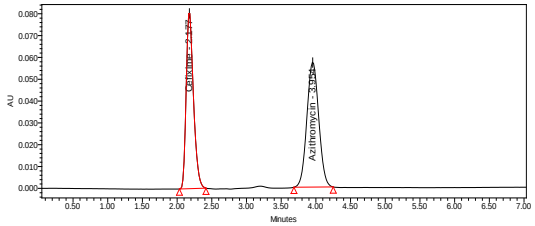

Figure.10.Chromatogram of sample

Inference: Got a peak for sample at an Rt of 2.177min for Cefixime and 3.954min for Azithromycin

Observation: The chromatograms of Standard and Sample were same identical with same retention time. As shown in fig: 9 and fig: 10

Precision:

System precision: Standard solution prepared as per test method and injected five times.

Method precision: Prepared six sample preparations individually using single as per test method and injected each solution.

Acceptance criteria: The % relative standard deviation of individual Cefixim and Azithromycin, from the six units should be not more than 2.0%. The individual assays of Cefixim and Azithromycin should be not less than 98% and not more than 102.0%.

Observation: Test results are showing that the test method is precise. Refer tables 2 and 3 for system precision and for method precision.

Repeatability:

(a) System precision:

Table.2(a).Data of Repeatability (System precision) for Cefixime

	Injection	Peak Areas of Cefixime	%Assay
Concentration 40ppm	1	2978965	100.51
	2	2970867	100.24
	3	2973742	100.34
	4	2978761	100.51
	5	2978642	100.50
Statistical **Analysis**	**Mean**	2976195	100.42
	SD	3696.277	0.123895
	% RSD	0.124195	0.123377

Table.2(b).Data of Repeatability (System precision) for Azithromycin

	Injection	Peak Areas of Azithromycin	%Assay
Concentration 40ppm	1	429853	100.34
	2	429741	100.31
	3	429784	100.32
	4	429403	100.23
	5	429746	100.31
Statistical **Analysis**	**Mean**	429705.4	100.302
	SD	174.8751	0.04207
	% RSD	0.040697	0.041945

(b)Method precision:

Table.3(a).Data of Repeatability (Method precision) for Cefixime

	Injection	Peak Areas of Cefixime	%Assay
Concentration 40ppm	1	2970873	100.24
	2	2978461	100.50
	3	2978462	100.50
	4	2978462	100.50
	5	2976542	100.43
	6	2978642	100.50
Statistical **Analysis**	**Mean**	2976907	100.445
	SD	3059.528	0.104259
	% RSD	0.0102775	0.103797

Table.3(b).Data of Repeatability (Method precision) for Azithromycin

	Injection	Peak Areas of Azithromycin	%Assay
Concentration 40ppm	1	429827	100.33
	2	429391	100.23
	3	429085	100.56
	4	429786	100.32
	5	429375	100.23
	6	429274	100.20
Statistical Analysis	**Mean**	429456.3	100.3117
	SD	292.606	0.132577
	% RSD	0.068134	0.132165

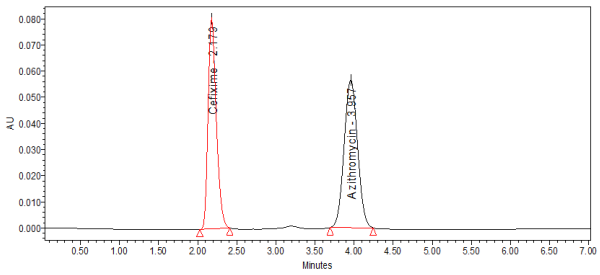

Figure.11.Chromatogram for Repeatability (standard - 1)

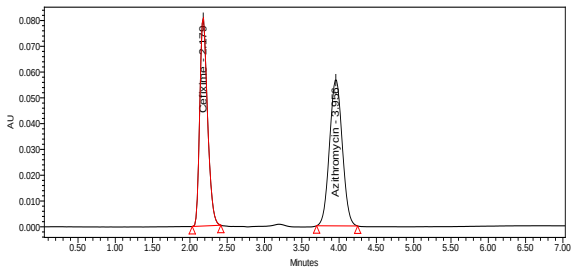

Figure.12.Chromatogram for Repeatability (standard - 2)

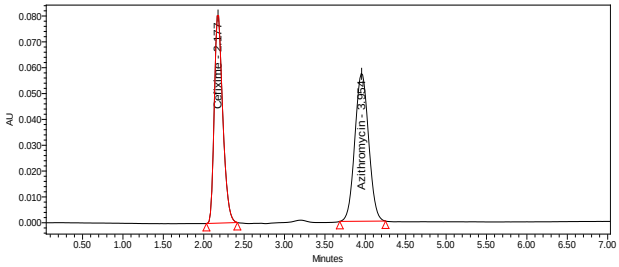

Figure.13.Chromatogram for Repeatability (standard - 3)

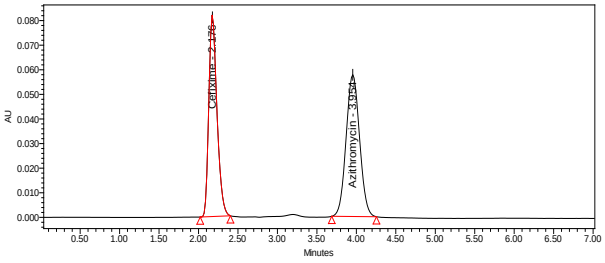

Figure.14.Chromatogram for Repeatability (standard - 4)

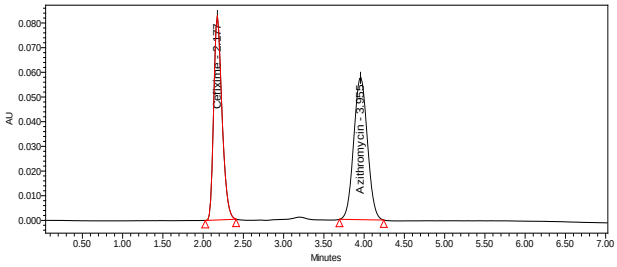

Figure.15.Chromatogram for Repeatability (standard - 5)

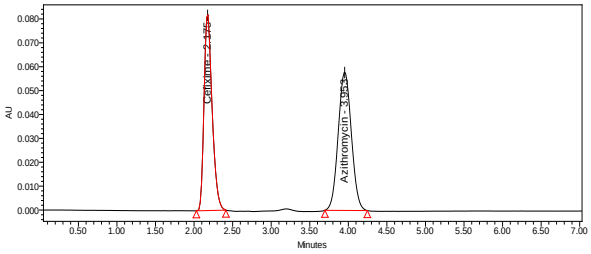

Figure.16.Chromatogram for Repeatability (standard - 6)

Intermediate precision (analyst to analyst variability): A study was conducted by two analysts as per test method
Acceptance criteria: The individual assays of Cefixime and Azithromycin should be not less than 98% and not more than 102% and %RSD of assays should be NMT2.0% by both analysts.
Intermediate precision:

Table.4(a).Data of Intermediate precision (Analyst 2) for Cefixime

	Injection	Peak Areas of Cefixime	%Assay
Concentration 40ppm	1	2970478	100.23
	2	2978492	100.50
	3	2970874	100.24
	4	2977892	100.48
	5	2970845	100.24
	6	2978632	100.50
Statistical **Analysis**	**Mean**	2974536	100.365
	SD	4175.907	0.140819
	% RSD	0.140389	0.140307

Table.4(b).Data of Intermediate precision (Analyst 2) for Azithromycin

	Injection	Peak Areas of Azithromycin	%Assay
Concentration 40ppm	1	429874	100.34
	2	429654	100.29
	3	429658	100.29
	4	429631	100.29
	5	429874	100.34
	6	429631	100.34
Statistical **Analysis**	**Mean**	429720.3	100.315
	SD	119.5603	0.027386
	% RSD	0.027823	0.0273

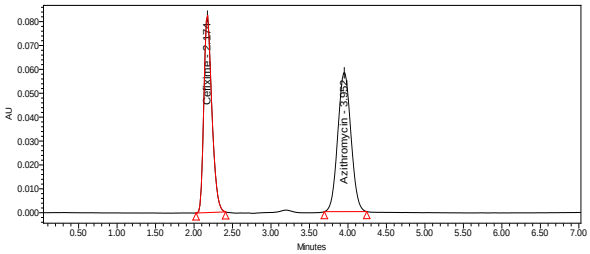

Figure.17.Chromatogram for Intermediate Precision

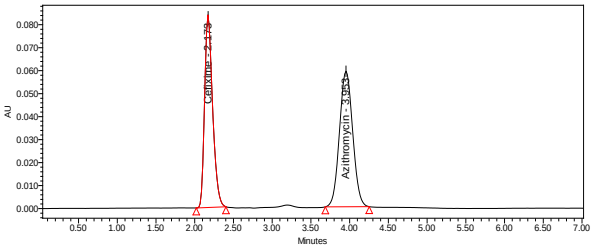

Figure.18.Chromatogram for Intermediate Precision

Observation: Individual %assays and %RSD of Assay are within limit and passes the intermediate precision, Refer table: 4

Accuracy (recovery): A study of Accuracy was conducted. Drug Assay was performed in triplicate as per test method with equivalent amount of Cefixime and Azithromycin into each volumetric flask for each spike level to get the concentration of Cefixime and Azithromycin equivalent to 50%, 100%, and 150% of the labeled amount as per the test method. The average % recovery of Cefixime and Azithromycin were calculated.

Acceptance criteria: The mean % recovery of the Cefixime and Azithromycin at each spike level should be not less than 98.0% and not more than 102.0% for both the drugs separately.

Observation:

$$\%\ \boldsymbol{Recovery} = \frac{\boldsymbol{Amount\ found}}{\boldsymbol{Amount\ added}} \times \mathbf{100}$$

The recovery results indicating that the test method has an acceptable level of accuracy.

Table.5(a).Data of Accuracy for Cefixime

Concentration % of spiked level	Amount added (ppm)	Area	Amount found (ppm)	% Recovery	Statistical Analysis of % Recovery	
50% Injection 1	20	1486721	19.89	99.47	**MEAN**	99.5433
50% Injection 2	20	1489764	19.94	99.68		
50% Injection 3	20	1486795	19.90	99.48	**%RSD**	0.119006
100 % Injection 1	40	2978864	40.20	100.51	**MEAN**	100.4633
100 % Injection 2	40	2976794	40.18	100.44		
100% Injection 3	40	2976874	40.18	100.44	**%RSD**	0.040228
150% Injection 1	60	4465274	60.44	100.73	**MEAN**	100.783
150% Injection 2	60	4467892	60.47	100.79		
150% Injection 3	60	4469874	60.50	100.83	**%RSD**	0.049941

Table.5(b).Data of Accuracy for Azithromycin

Concentration % of spiked level	Amount added(ppm)	Area	Amount found (ppm)	% Recovery	Statistical Analysis of % Recovery	
50% Injection 1	20	214836	19.92	99.62	**MEAN**	99.593
50% Injection 2	20	214975	19.94	99.68		
50% Injection 3	20	214558	19.90	99.48	**%RSD**	0.103051
100 % Injection 1	40	429754	40.13	98.92	**MEAN**	99.66
100 % Injection 2	40	429634	40.11	99.75		
100% Injection 3	40	429754	40.13	100.31	**%RSD**	0.701743
150% Injection 1	60	644876	60.35	99.96	**MEAN**	100.3467
150% Injection 2	60	644968	60.36	100.59		
150% Injection 3	60	644308	60.29	100.49	**%RSD**	0.007344

Linearity of test method: A Series of solutions are prepared using Cefixime and Azithromycin working standards at concentration levels from 20ppm to 80 ppm of target concentration. Measure the peak area response of solution at Level 1 and Level 6 six times and Level 2 to Level 5 two times.

Acceptance criteria: Correlation Coefficient should be not less than 0.9990.

% of y- Intercept should be ±2.0.

% of RSD for level 1 and Level 6 should be not more than 2.0%.

Observation: The linear fit of the system was illustrated graphically.

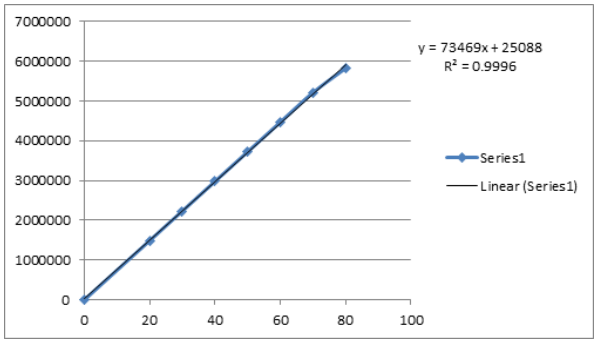

Figure.19.Linearity Plot (Concentration Vs Response) of Cefixime

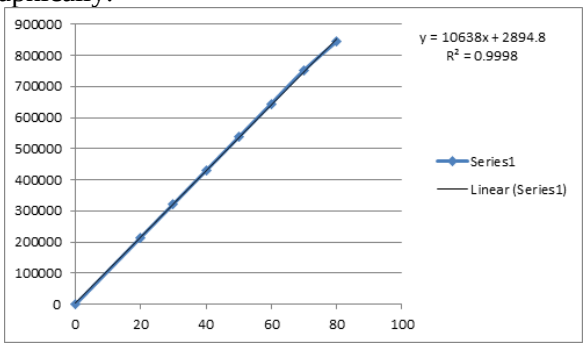

Fig: 52 Linearity Plot (Concentration Vs Response) of Azithromycin

Ruggedness of test method:

a) System to system variability: System to system variability study was conducted on different HPLC systems, under similar conditions at different times. Six samples were prepared and each was analyzed as per test method. Comparison of both the results obtained on two different HPLC systems, shows that the assay test method are rugged for System to system variability.

Acceptance criteria: The % relative standard deviation of Cefixime and Azithromycin from the six sample preparations should be not more than 2.0%

The % assay of Cefixime and Azithromycin should be between 98.0%-102.0%.

Observation: The % RSD was found within the limit.

Table.6(a).Data of system to system variability (Cefixime) System-2

S.NO	Peak area	Assay % of Cefixime
1	2975974	100.41
2	2978542	100.50
3	2978792	100.51
4	2970875	100.23
5	2978453	100.50
6	2971542	100.26
Mean	2975696.33	100.4017
%RSD	0.121967	0.126522

Table.6(b).Data of system to system variability (Azithromycin) System-2

S.NO	Peak area	Assay % of Azithromycin
1	429871	100.34
2	429637	100.29
3	429654	100.34
4	429875	100.34
5	429875	100.34
6	428754	99.08
Mean	429611.	100.121
%RSD	0.101139	0.5100815

Observation: The results obtained by comparing with both two types were within limit.

Robustness:

a) Effect of variation of flow rate: A study was conducted to determine the effect of variation in flow rate. Standard solution prepared as per the test method was injected into the HPLC system using flow rates, 1.0ml/min and 1.2ml/min. The system suitability parameters were evaluated and found to be within the limits for 1.0ml/min and 1.2ml/min flow.

Cefixim and Azithromycin was resolved from all other peaks and the retention times were comparable with those obtained for mobile phase having flow rates 1.0ml/min.

Acceptance criteria: The Tailing Factor of Cefixim and Azithromycin standards should be NMT 2.0 for Variation in Flow.

Table.7(a).Data for Effect of variation in flow rate (Cefixime)

Flow 0.8 ml	Std Area	Tailing factor	Flow 1.0 ml	Std Area	Tailing factor	Flow 1.2 ml	Std Area	Tailing factor
	2968201	1.099372		2978431	1.128451		2984051	1.121875
	2958708	1.103587		2976848	1.112257		2986371	1.122254
	2968754	1.111587		2978462	1.121287		2983078	1.124357
	2965882	1.117861		2970894	1.124752		2987628	1.123895
	2962082	1.119547		2978452	1.123874		2986071	1.099157
Avg	2964725	1.110391	**Avg**	2976617	1.122124	**Avg**	2985440	1.118308
SD	4267.429	0.008786	**SD**	3273.68	0.006084	**SD**	1841.238	0.010757
%RSD	0.14394	0.791255	**%RSD**	0.10998	0.542182	**%RSD**	0.061674	0.961908

Table.7(b).Data for Effect of variation in flow rate (Azithromycin)

Flow 0.8 ml	Std Area	Tailing factor	Flow 1.0 ml	Std Area	Tailing factor	Flow 1.2 ml	Std Area	Tailing factor
	428631	1.238915		429860	1.251658		430584	1.262276
	428894	1.230637		429631	1.245435		430963	1.251581
	428634	1.240858		429874	1.262464		430217	1.237875
	428761	1.238995		429364	1.237018		430492	1.239824
	428637	1.241073		429874	1.239010		430674	1.238257
Avg	429711.4	1.238096	**Avg**	429720.6	1.247117	**Avg**	430586	1.245963
SD	115.9668	0.00429	**SD**	225.548	0.010328	**SD**	271.5115	0.010726
%RSD	0.02705	0.346477	**%RSD**	0.052254	0.828172	**%RSD**	0.063056	0.860835

Observation: The tailing factor for Cefixime and Azithromycin was found to be within the limits. As shown in table 8.

Effect of variation of temperature: A study was conducted to determine the effect of variation in temperature. Standard solution prepared as per the test method was injected into the HPLC system at 20ºC temperature. The system suitability parameters were evaluated and found to be within the limits for a temperature change of 20ºc.

Similarly sample solution was chromatographed at 25ºC temperature. Cefixime and Azithromycin were resolved from all other peaks and the retention times were comparable with those

Acceptance criteria: The Tailing Factor of Cefixime and Azithromycin standard and sample solutions should be NMT 2.0 for Variation in temperature.

Limit of detection and quantitation (LOD and LOQ): From the linearity data calculate the limit of detection and quantitation, using the following formula.

$$LOD = \frac{3.3\sigma}{S}$$

σ = standard deviation of the response

S = slope of the calibration curve of the analyte

$$LOQ = \frac{10\sigma}{S}$$

σ = standard deviation of the response

S = slope of the calibration curve of the analyte

Cefixime: From the linearity plot the LOD and LOQ are calculated:

$$LOD = \frac{3.3\sigma}{S} = \frac{3.3 \times 2124.413}{20193} = 0.34$$

$$LOQ = \frac{10\sigma}{S} = \frac{10 \times 2124.413}{20193} = 1.05$$

Azithromycin:

$$LOD = \frac{3.3\sigma}{S} = \frac{3.3 \times 2431.578}{31282} = 0.25$$

$$LOQ = \frac{10\sigma}{S} = \frac{10 \times 2431.578}{31282} = 0.77$$

3. RESULTS AND DISCUSSION

A simple, economic, accurate and precise reverse phase high performance liquid chromatography method was developed for analysis of Cefixime and Azithromycin crude drug and its marketed formulation. Method developed and validated according to ICH guidelines. The quantification of the drug was carried out using Waters 2690 HPLC instrument and PDA (photodiode array) detector. Column is isocratic mode.

Results for chromatographic conditions are:

Mobile phase : Methanol: Buffer (ratio 85:15)
Column : Hypersil C_{18} column (250 x4.6, size: 5µm)
Detector : Photodiode array detector
Mode : Isocriatic
Flow Rate : 1.0 mL/min
Column Temperature : Ambient (25^0C)
Wave length : 275nm
Injection Volume : 20 µL
Run Time : 10 min

Validation data:

System suitability: The system suitability parameters were evaluated and found to be within the limits. The % RSD for peak areas from six replicate injections of Cefixime and Azithromycin was found to be 0.10% and0.07%.

Specificity: There is no interference at Retention Time of Cefixime and Azithromycin peaks

Precision: The precision of test procedure was evaluated for Cefixime and Azithromycin by performing the assay as per the test method for six times. The Recovery of Cefixime and Azithromycin & % RSD for peak area was found to be within the limits and it was 0.1241&0.0406 respectively.

Linearity of detector response: The linearity data was obtained from the above graph using concentration vs Peak area. The linearity of the Cefixime and Azithromycin was found to be 0.999. The acceptance criteria of the linearity is 0.999

Accuracy: Accuracy was conducted by performing the recovery studies of Cefixime and Azithromycin raw drug samples at 50%, 100% and 150% spiked levels in triplicate. The average % recovery of Cefixime and Azithromycin was found to be within the limits and that are 100.26-99.86 respectively.

Robustness: Robustness was conducted by deliberately changing the flow rate (0.2ml/min) and temperature we can calculate the robustness. The developed method retain its reliability by getting % RSD value (for peak area) of Cefixime and Azithromycin was found to be 1.11%, 1.23%

Limit of Detection: It was found to be 0.34&0.25 (acceptance criteria of LOD is below1)

Limit of Quantification: It was found to be 1.05&0.77. (Acceptance criteria of LOQ 3times to the LOD value)

Table.8.Optimized parameters

Parameter	Results	
	Cefixime	**Azithromycin**
System suitability(%RSD)	0.10%	0.07%
Specificity	No interference of any peaks	No interference of any peaks
Correlation coefficient(r^2)	0.999	0.999
Intercept	25088	2894
Slope	73469	10638
Precession(%RSD)	0.1241	0.0406
Accuracy	100.26	99.86
Limit of detection(LOD)	0.34	0.25
Limit of quantification(LOQ)	1.05	0.77
Robustness	1.11	1.23

4. CONCLUSION

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found for Cefixime at 237nm and 275nm for Azithromycin. Common wavelength will be 275nm and the peaks purity was excellent. Injection volume was selected to be 20µl which gave a good peak area. The column used for study was Inertsil C_{18}, ODS chosen good peak shape. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area, satisfactory retention time and good resolution. Different ratios of mobile phase were studied, mobile phase with ratio of 85:15 Methanol : Buffer was fixed due to good symmetrical peaks and for good resolution. So this mobile phase was used for the proposed study.

The present recovery was found to be 98.0-101.50 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range. Detection limit was found to be 0.25 Cefixime and 0.34 for Azithromycin. Linearity study was, correlation coefficient and curve fitting was found to be. The analytical method was found linearity over the range of 20-80ppm of the target concentration for both the drugs. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

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