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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR AZITHROMYCIN AND LEVOFLOXACIN COMBINED TABLET DOSAGE FORM

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ABSTRACT

The aim of the study is an attempt has been made to develop simultaneous determination methods for combined dose tablet formulation Azithromycin and Levofloxacin the by a simple, accurate, precise, sensitive, less expensive and less time consuming method by using RP-HPLC in pharmaceutical dosage form. The method was validated for parameters like accuracy, linearity, precision, specificity, robustness and system suitability. The column efficiency as determined is not less than 3000 USP plate count and the tailing factor is not more than 2.0. The % relative standard deviation for the peak areas of the six replicate injections is not more than 2.0%. The % RSD of assay of six replicate injections was found to be within the limits. The recovery results indicating that the test method has an acceptable level of accuracy. The correlation coefficient met the acceptance criteria of NLT 0.999. The LOD and LOQ values from the study demonstrate that the method is sensitive. The system suitability parameters found to be within the limits for a temperature change of 200^0C, 250^0C, 300^0C. Similarly sample solution was chromate graphed at 200^0C, 250^0C and 300^0C temperature. Retention times were compared and were found that with the increase in temperature retention time decreases. A study was conducted to determine the effect of variation in flow rate and from the results it is concluded that the method is robust.

KEY WORDS

Azithromycin, Levofloxacin, RP-HPLC, Retention time and ICH guideline.

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INTRODUCTION

Analytical chemistry is often described as the area of chemistry responsible for characterizing the composition of matter, both qualitatively (what is present) and quantitatively (how much is present). Analytical chemistry is not a separate branch of chemistry, but simply the application of chemical knowledge. Quantitative analysis constitutes the largest part of analytical chemistry and is related to

the various methods and instrumentation employed in determining the amounts or concentration of constituents in samples. It is also one of the basic criteria in the field of pharmacy where quality is to be critically maintained. Analytical chemistry may be defined as the "Science and art of determining the composition of materials in terms of the elements or compounds contained". Analytical method is a specific application of a technique to solve an analytical problem[1]. High-performance liquid chromatography (HPLC) is a form of liquid chromatography to separate compounds that are dissolved in solution. HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector.

Compounds are separated by injecting a plug of the sample mixture into the column. The different components in the mixture pass through the column at different rates due to differences in their partition behavior between the mobile liquid phase and the stationary phase[2]. High performance liquid chromatography (HPLC) is the fastest growing analytical technique for the analysis of drugs. Its simplicity, high specificity and wide range of sensitivity makes it ideal for the analysis of many drugs in both dosage forms and biological fluids. In HPLC the separation is about 100 times faster than the conventional liquid chromatography due packing of stationary phase particles in the range of 5-10μm[3].

MATERIAL AND METHOD

MATERIAL

Levofloxacin and Azithromycin were gifted from Medico remedies pvt.ltd, India. Di-potassium hydrogen phosphate, Methanol, Losartan Potassium were gifted from Qualigens fine chemicals Mumbai, India. All other materials used in the study were of analytical or pharmaceutical grade.

METHOD

Method validation can be defined as (ICH) Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics. All the variables of the method should be consider, including sampling procedure, sample preparation, chromatographic separation, and detection and data evaluation. The ICH has published specific guidelines for method validation for compound evaluation. ICH defines eight steps for validation: Accuracy, Precision, Specificity, Limit of detection, and Limit of quantitation, Linearity and range, Robustness[4].

Analytical Procedure

The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to: the sample, the reference standard and the reagents preparations, use of the apparatus generation of the calibration curve, use of the formulae for the calculation, etc[5].

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

This is sometimes termed trueness. Accuracy is best report as percentage bias, which is calculated from the expression

$$\text{Accuracy} = \frac{\text{(Measured value – True value)}}{\text{True value}} \times 100$$

Since for real samples the true value is not known, an approximation is obtained based on spiking drug-free matrix to a normal concentration. The accuracy of analytical method is then determined at each concentration by assessing the agreement between the measured and nominal concentrations of the analyte in the spiked drug- free matrix sampler[6].

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. The precision of an analytical procedure is usually expressed as the percentage coefficient of variation (% CV), or relative standard deviation (RSD) of the replicate measurements.

$$\% CV = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

Precision can be considered as having a within assay batch component or repeatability which defines the ability to repeat the same methodology with the same analyst, using the same equipment and same reagents in a short interval of time, eg. Within a day. The ability to repeat the same methodology under different conditions, eg. change of analyst, reagent or equipment, or on subsequent occasions, eg across several weeks or months, is covered by the between batch precision or reproducibility, also known as inter-assay precision. The reproducibility of the method gives better representation of the precision during routine use as it includes the variability from many sources.

Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s). This definition has the following implications: Identification- To ensure the identity of an analyte.

Purity Tests

To ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

Assay (content or potency)

To provide an exact result this allows an accurate statement on the content or potency of the analyte in a sample.

Detection Limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantities as an exact value. It may be defined as the concentration, which gives rise to an instrumental signal that is significantly different from the blank. For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (Sa), which may be related to LOD and the slope of the calibration curve , b, by LOD = 3 Sa/b.

Quantitation Limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

The LOQ represent the concentration of analyte that would yield a signal- to- noise ratio of 10^7.

$$LOQ=10\ Sa/b$$

Where Sa- the estimate is the standard deviation of the peak are ratio of analyte to IS (5 injections) of the drugs is slope of the corresponding calibration curve.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage such as ph of the mobile phase, temperature, %organic solvent strength and buffer concentration etc. to determine the robustness of the method experimental conditions were purposely altered and chromatographic characters were evaluated.

OPTIMIZED METHOD

Reference standards: Azithromycin and Levofloxacin.

Strengths of dosage forms: Azithromycin - 500 mg, Levofloxacin - 500 mg.

Preparation of mobile phase

Mix a Di Potassium Hydrogen Phosphate (60%) and methanol (HPLC grade) (40%) and degas in ultrasonic water bath for 15 minutes. Filter through 0.45 µ filter under vaccum filtration.

Standard stock solution preparation

Accurately weigh and transfer 500mg of Azithromycin and 500mg of Levofloxacin working standard into 100 ml volumetric flask add about 50mL of Diluent(Mobile phase and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).

Standard solution preparation

From the above stock solution take 5ml in 50 ml volumetric flask and make up the volume with diluents.

Sample stock solution preparation

Accurately weigh and transfer equivalently 500 mg of sample to clean dry 100 ml volumetric flask and add the 50ml volume to the mark with diluents (Mobile phase) and sonicate to dissolve it completely and make volume up to the mark with the same solvent

Sample solution preparation

From the above stock solution take 5ml in 50 ml volumetric flask and make up the volume with diluent.

Evaluation of System Suitability

Inject 10 µl of the diluted standard solution in five replicate injections, into the chromatograph and record the chromatograms. The column efficiency as determined from Azithromycin and

Levofloxacin peaks is not less than 3000 USP plate count and the tailing factor for Azithromycin and Levofloxacin peaks is not more than 2.0. The relative standard deviation for the peak areas of the five replicate injections is not more than 2.0%[8].

Procedure

Separately inject 10µl of the blank, Standard (five injections) and sample solution in duplicate into the liquid chromatography, record the chromatographs and measure the peak areas.

Calculation

Calculate the amount of each drug by using the following formula.

$$(\text{mg/tablet}) = \frac{A_L}{A_{SL}} \times \frac{Ds}{D_T} \times \frac{P}{100}$$

Where,

AL = Average area counts of injections for analyte peak in the chromatogram of sample solution.

ASL = Average area count of five replicate injections for analyte peak in the chromatogram of standard solution.

DS = Dilution factor of standard solution (weight/dilution).

DT = Dilution factor of sample solution.

P = Percentage purity of working standard used.

$$\% \text{ Labeled Amount} = \frac{\text{Content of each drug (mg/tablet)}}{\text{Label claim, in mg}} \times 100$$

RP-HPLC Method development

A simple reverse phase HPLC method was developed for estimation of Azithromycin and Levofloxacin in their pharmaceutical formulation. A Waters symmetry shield Rp18, (250x4.6x5µ column, along with Di Potassium Hydrogen Phosphate and methanol in the ratio of 60:40 as mobile phase. The flow rate was 1.0 ml/min and effluent was monitored at 285 nm.

The retention times were 5.001 min and 3.232 min for Azithromycin and Levofloxacin respectively. Analysis of drugs present in pharmaceutical dosage

forms is a quite challenging problem and hence attempts were made to develop analytical methods for simultaneous estimation of Azithromycin and Levofloxacin in Pharmaceutical dosage forms[9].

All the proposed methods are simple, selective, reproducible, sensitive and accurate with good precision. Some of the methods were proved to be superior to most of the reported methods. All these proposed methods for estimation of Azithromycin and Levofloxacin were successfully applied in pharmaceutical formulations.

The proposed method can be used as alternative methods to the reported ones for the simultaneous estimation of Azithromycin and Levofloxacin. Thus the purpose of the present investigation was successfully achieved[10].

RESULTS AND DISCUSSION

Analytical Method Development: Selection of mobile phase

Several solvent systems were tried to get good optimum resolutions of Losartan Potassium and Hydrochlorothiazide in the chromatogram. The chromatogram is presented below.

Phosphate

Methanol in the ratio of (60: 40, v/v)

It was found that peaks of Azithromycin and Levofloxacin were well resolved with the solvent system of di-Potassium hydrogen Phosphate: Methanol in the ratio of (60: 40, v/v).

Method Validation Parameters

System suitability

System suitability tests are used to verify the reproducibility of the chromatographic system. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared stock solutions.

Method precision

The % RSD of six replicate injections of Azithromycin standard preparation is within the specified acceptance criteria. The % RSD of six replicate injections of Levofloxacin standard preparation is within the specified acceptance criteria.

Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value.

Accuracy 150%

Linearity

Specificity

The chromatograms of Azithromycin and hydrochloro thiazide were analyzed and there is no interference from diluents, excipients with peaks of Azithromycin and Levofloxacin.

Limit of Detection

Lowest amount of analyte that can be detected but not necessarily quantitated. It was calculated from signal to noise ratio.3.

The LOQ values from the above demonstrate that the method is sensitive for the determination of Azithromycin and Levofloxacin.

Robustness

All the results are tabulated and showed in Table No.1 to 15 and Figure No.1 to 10.

SUMMARY

Development of new analytical methods for the determination of drugs in pharmaceutical dosage forms is more important in pharmacokinetic, toxicological and biological studies. Today pharmaceutical analysis entails much more than the analysis of active pharmaceutical ingredients or the formulated product. The pharmaceutical industry is under increased scrutiny from the government and the public interested groups to contain costs and at consistently deliver to market safe, efficacious product that fulfill unmet medical needs. The pharmaceutical analyst plays a major rule in assuring identity, safety, efficacy, purity, and quality of a drug product. The need for pharmaceutical analysis is driven largely by regulatory requirements. The commonly used tests of pharmaceutical analysis generally entail compendia testing method development, setting specifications, and method validation. Analytical testing is one of the more interesting ways for scientists to take part in quality process by providing actual data on the identity, content and purity of the drug products.

New methods are now being development with a great deal of consideration to worldwide harmonization. As a result, new products can be assured to have comparable quality and can be brought to international markets faster[11].

Table No.1: Optimized Chromatographic conditions

S.No	Stationary phase (column)	A Waters symmetry shield Rp18, (250x4.6x5) µ column
1	Mobile phase	Di-potassium hydrogen phosphate: methanol (60:40)
2	Ph	9.2
3	Flow rate (ml/min)	1ml/min
4	Run time (minutes)	7 mins
5	Column temperature (0C)	30^0C
6	Volume of injection loop (µl)	10
7	Detection wavelength(nm)	285nm
8	Drug	Azithromycin and Levofloxacin
9	Drug Rt (min)	5.001 and 3.232

Table No.2: System suitability Parameters for Azithromycin

S.No	System Suitability Parameter	Result obtained	Acceptance Criteria
1	% RSD for six replicate injections of analyte peak in standard solution	0.6	NMT 2.0
2	Tailing factor for analyte peak in standard solution	1.213	NMT 2
3	USP Plate count of Analyte peak of standard solution	6782	NLT 3000

Table No.3: System suitability Parameters for Levofloxacin

S.No	System Suitability Parameter	Result obtained	Acceptance Criteria
1	% RSD for six replicate injections of analyte peak in standard solution	0.7	NMT 2.0
2	Tailing factor for analyte peak in standard solution	1.105	NMT 2
3	USP Plate count of Analyte peak of standard solution	4670	NLT 3000

Table No.4: Results of Azithromycin for precision studies

S.No	Retention Time	Peak Area	Assay %
1	5.005	3503557	99.72
2	5.007	3505877	99.79
3	5.006	3503969	99.73
4	5.006	3508933	99.87
5	5.006	3501948	99.67
6	5.004	3501546	99.66
% RSD		---	0.08

Table No.5: Results of Levofloxacin for precision studies

S.No	Retention time	Peak area	Assay %
1	3.234	3152466	99.10
2	3.233	3151745	99.08
3	3.231	3158954	99.31
4	3.231	3157787	99.27
5	3.229	3158663	99.30
6	3.230	3155433	99.19
% RSD		---	0.10

Table No.6: Results for Accuracy of Azithromycin

S.No	Concentration	Retention Time	Peak area	% Recovery	Mean Recovery
1	50	5.009	1751636	99.87	
2	50	5.009	1751270	99.85	
3	50	5.003	1758366	100	
4	50	5.003	1756333	100	
5	50	4.995	1750913	99.83	99.89
6	50	4.991	1750708	99.81	
7	100	4.982	3508264	100	
8	100	4.978	3509457	100	99.98
9	100	4.977	3506370	99.95	
10	150	4.971	5253135	99.59	
11	150	4.972	5250130	99.53	
12	150	4.971	5251467	99.56	
13	150	4.974	5259637	99.71	
14	150	4.973	5255756	99.64	99.6
15	150	4.968	5253042	99.59	

Table No.7: Results for Accuracy of Levofloxacin

S.No	Concentration	Retention Time	Peak area	% Recovery	Mean Recovery
1	50	3.236	1579162	100	99.75
2	50	3.236	1576213	99.85	
3	50	3.231	1573695	99.69	
4	50	3.233	1570450	99.49	
5	50	3.232	1576050	99.84	
6	50	3.230	1573005	99.65	
7	100	3.222	3152275	99.85	99.89
8	100	3.220	3151260	99.82	
9	100	3.220	3157232	100	
10	150	3.216	4726940	99.58	99.58
11	150	3.216	4729848	99.64	
12	150	3.216	4723140	99.50	
13	150	3.216	4728536	99.61	
14	150	3.217	4728895	99.62	
15	150	3.210	4724036	99.51	

The % Recovery at each level is between 98.0% to 102.0%

Table No.8: Linearity Studies of Azithromycin

S.No	Sample Name	Inj	Name	RT	Area
1	LINEARITY-50%	1	Azithromycin	4.982	1750331
2	LINEARITY-75%	1	Azithromycin	4.978	2621620
3	LINEARITY-100%	1	Azithromycin	4.977	3501905
4	LINEARITY-125%	1	Azithromycin	4.975	4374337
5	LINEARITY-150%	1	Azithromycin	4.976	5253057

Table No.9: Linearity Studies of Levofloxacin

S.No	Sample Name	Inj	Name	RT	Area
1	LINEARITY-50%	1	Levofloxacin	3.223	1544038
2	LINEARITY-75%	1	Levofloxacin	3.221	2364521
3	LINEARITY-100%	1	Levofloxacin	3.218	3156465
4	LINEARITY-125%	1	Levofloxacin	3.213	3974279
5	LINEARITY-150%	1	Levofloxacin	3.211	4720111

Table No.10: Results of linearity for Azithromycin

S.No	% Concentration	Area
1	50	1750331
2	75	2621620
3	100	3501905
4	125	4374337
5	150	5253057

Table No.11: Results of linearity for Levofloxacin

S.No	% Concentration	Area
1	50	1544038
2	75	2364521
3	100	3156465
4	125	3974279
5	150	4720111

Table No.12: Specificity representation

S.No	Peak Name	Retention Time
1	Diluent	No peaks are observed at retention time of main Peak
2	Placebo	No peaks are observed at the retention time of main peak
3	Main peak (Azithromycin)	Azithromycin- 5.001
4	Main peak (Levofloxacin)	Levofloxacin- 3.232

Table No.13: Results of LOD for Azithromycin and Levofloxacin

S.No	Drug	LOD (ppm)	S/n
1	Azithromycin	20.50	244.524
2	Levofloxacin	18.25	274.027

Table No.14: Results of Robustness for Azithromycin and Levofloxacin

S.No	Sample name	Inj	Name	Rt	Area	Resolution	Tailing	Plate count
1	Std 2(flow1)	1	Azithromycin	6.165	4351433	8.307	1.243	7701
2	Std 2(flow2)	1	Azithromycin	4.154	2883646	7.537	1.192	6211
3	Std 2 (Temp1)	1	Azithromycin	6.130	4351835	8.339	1.210	7487
4	Std 2(Temp2)	1	Azithromycin	4.150	2874923	7.577	1.190	5954
5	Std 2(flow1)	1	Levofloxacin	3.992	3941463	6.809	1.125	5203
6	Std 2(flow2)	1	Levofloxacin	2.688	3941463	5.495	1.109	4342
7	Std 2(Temp1)	1	Levofloxacin	3.962	3923126	4.529	1.136	5106
8	Std 2(Temp2)	1	Levofloxacin	2.686	2592639	4.508	1.100	4348

Table No.15: Summary report of HPLC validation

S.No	Validation Parameters	Acceptance Criteria	HPLC Results	
1	Precision	The % RSD of peaks obtained from the 6 replicate injections should be NMT 2.0%	**Azithromycin**	**Levofloxacin**
			0.08	0.10
2	Accuracy	The % recovery at each level shall be NLT 98.0% and NMT 102.0% of the added amount	**Azithromycin**	**Levofloxacin**
			100	99
3	Linearity	The Correlation coefficient shall be NLT 0.999	**Azithromycin**	**Levofloxacin**
			0.99	0.99
4	Robustness	All the system suitability parameters should pass for all the conditions.	The system suitability parameters passed for all the Conditions	
5	System Suitability	For 6 replicate injections	**Azithromycin**	**Levofloxacin**
		The %RSD NMT 2.0%	0.6	0.7
		Tailing factor NMT 2.0%	1.213	1.105
		Plate Count NLT 3000	6782	4670

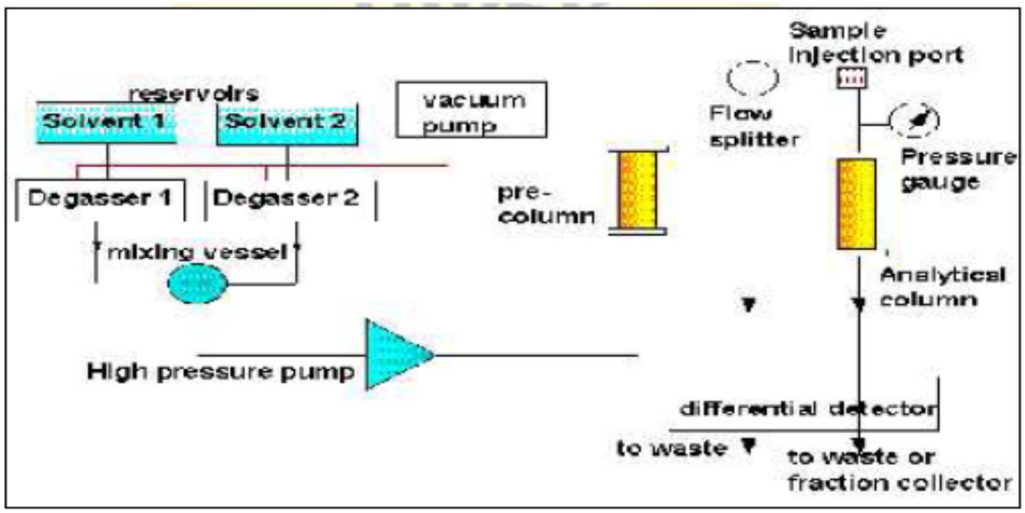

Figure No.1: Schematic diagram of an HPLC instrument

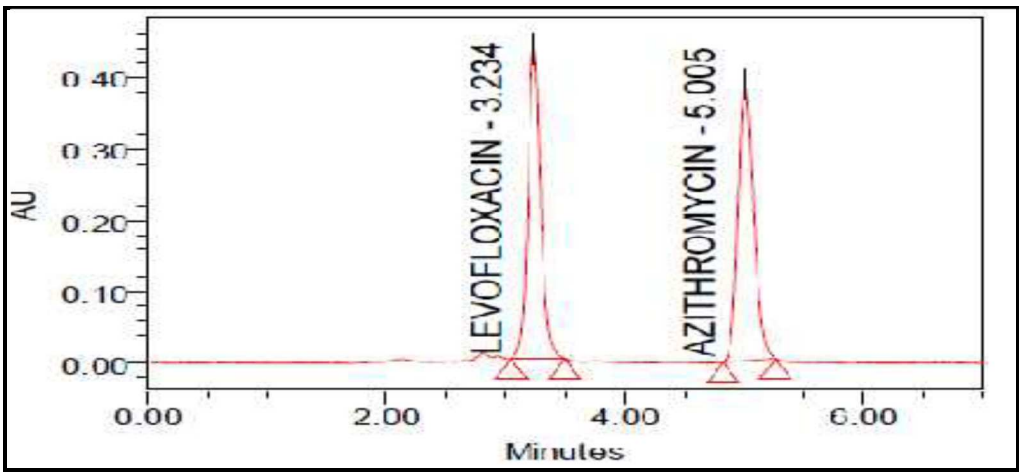

Figure No.2: Chromatogram of sample

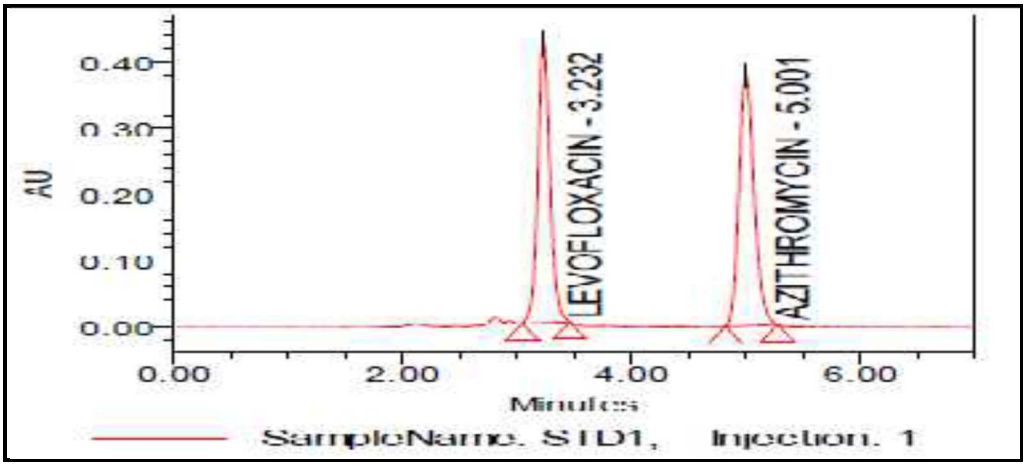

Figure No.3: Chromatogram showing peak separation with di- Potassium hydrogen

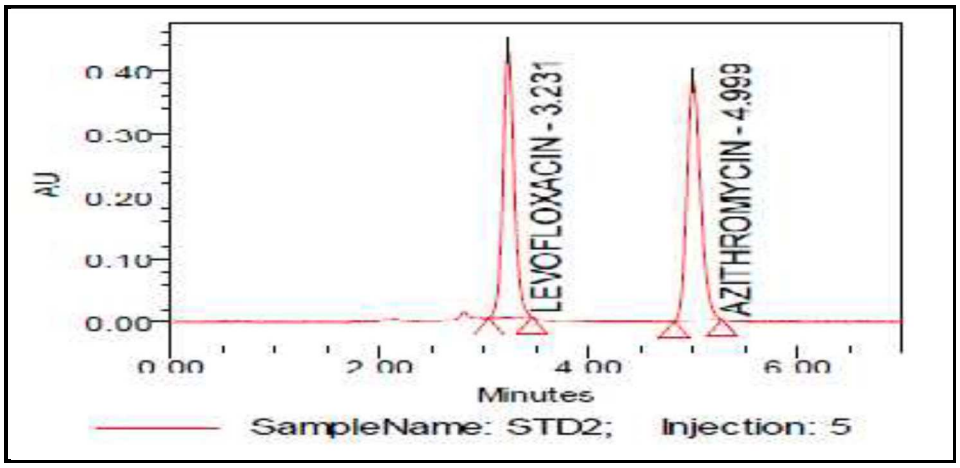

Figure No.4: System suitability chromatogram for Azithromycin and Levofloxacin

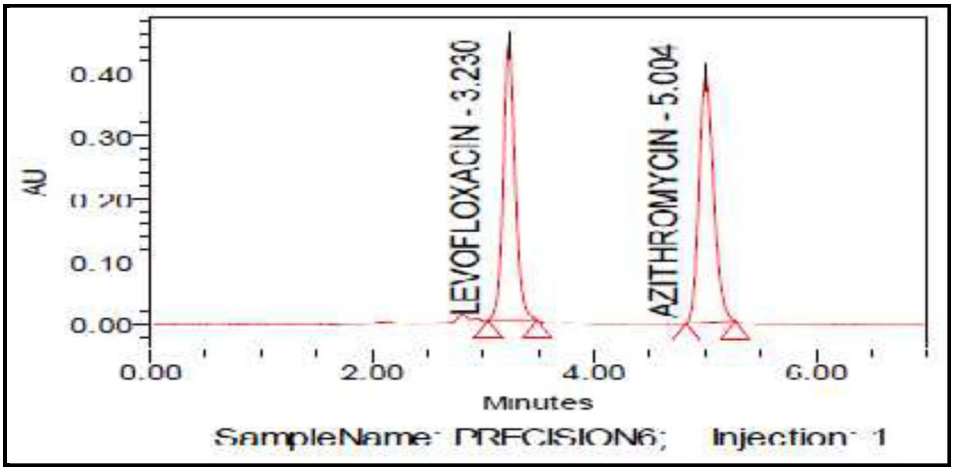

Figure No.5: Method Precision chromatogram of Azithromycin and Levofloxacin

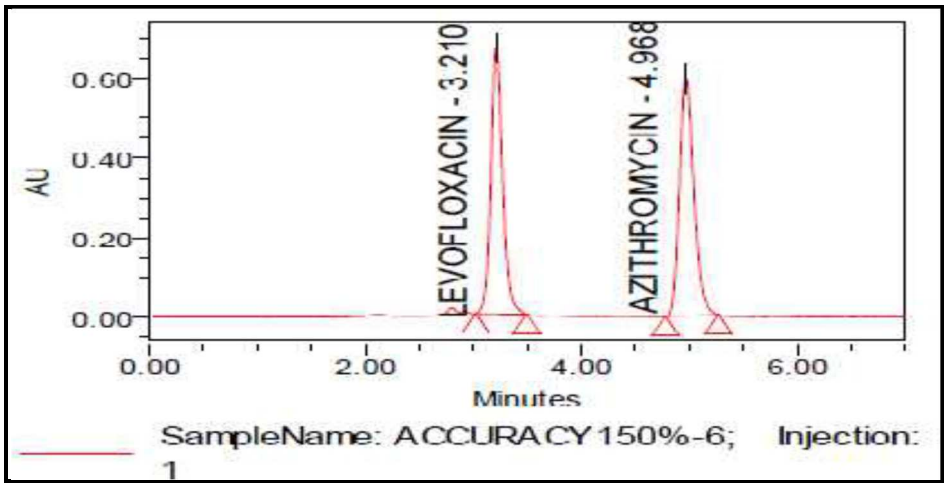

Figure No.6: Chromatograms of Accuracy studies

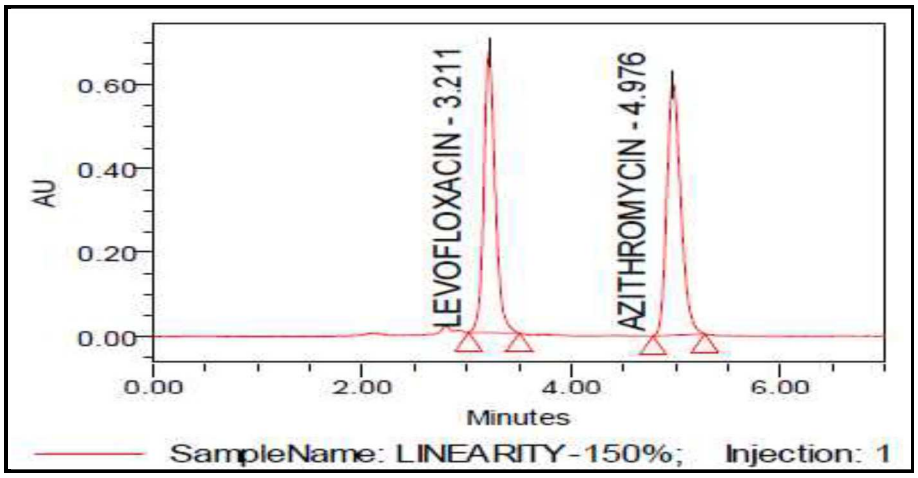

Figure No.7: Chromatogram of Linearity studies

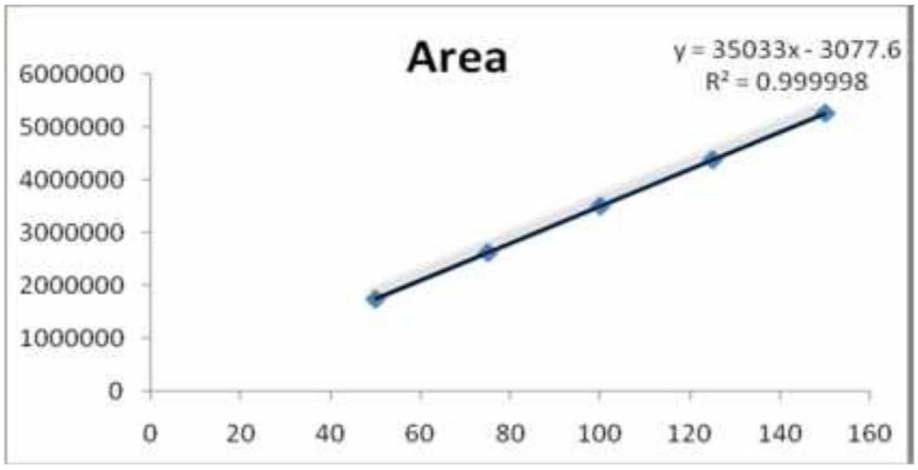

Figure No.8: Linearity plot of Azithromycin

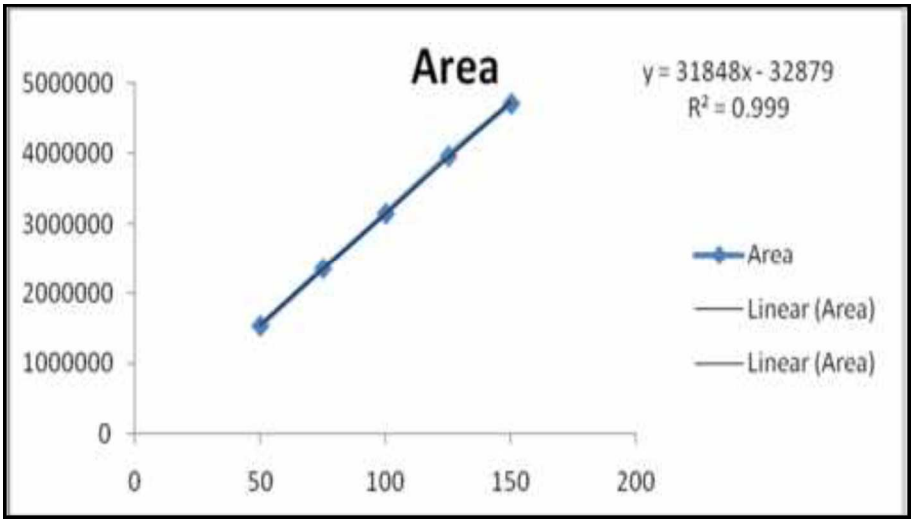

Figure No.9: Linearity plot of Levofloxacin

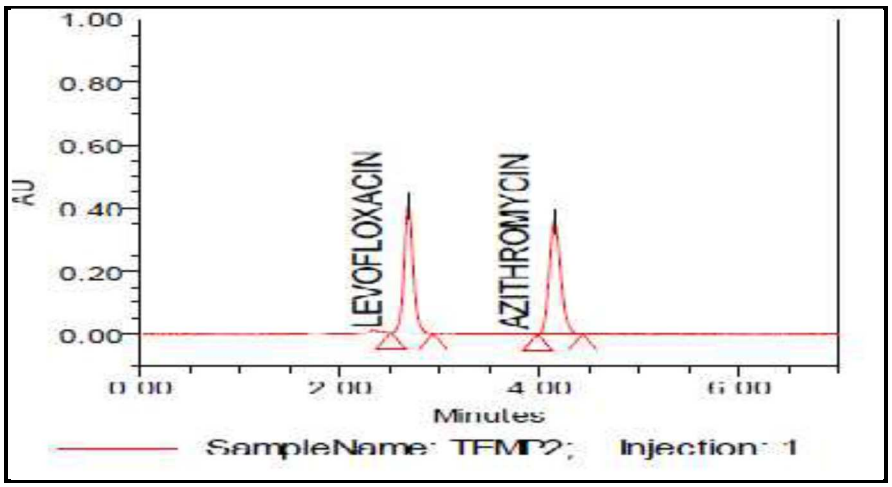

Figure No.10: Chromatograms of Robustness

CONCLUSION

Pharmaceutical analysis occupies a pivotal role in statuary certification of drugs and their formulations either by the industry or by the regulatory authorities. In industry, the quality assurance and quality control departments play major role in bringing out a safe and effective drug or dosage form. The current good manufacturing practices (CGMP) and the Food Drug Administration (FDA) guidelines insist for adoption of sound methods of analysis with greater sensitivity and reproducibility. Therefore, the complexity of problems encountered in pharmaceutical analysis with the importance of achieving the selectivity, speed, low cost, simplicity, sensitivity, specificity, precision and accuracy in estimation of drugs[12].

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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