EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 10
Issue: 2
307-315
Year: 2023

NEW SIMPLE STABILITY INDICATING LIQUID CHROMATOGRAPHY METHOD DEVELOPMENT AND VALIDATION FOR THE QUANTIFICATION OF PLAZOMICIN IN BULK DRUG SUBSTANCE AND PHARMACEUTICAL DOSAGE FORMS

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Article Received on 07/12/2022 **Article Revised on 28/12/2022** **Article Accepted on 18/01/2023**

ABSTRACT
Liquid chromatography is an analytical technique that is used to separate a certain sample into its individual components. HPLC is simple, specific, rapid, precise and accurate; it can be successfully and efficiently adopted for routine quality control analysis of drugs in bulk and pharmaceutical dosage form. In the present study a reverse phase high performance liquid chromatography method was developed and validated for the estimation Plazomicin in pharmaceutical formulations. To assess the effect of method parameters on chromatographic separation of the Plazomicin, statistically designed experiments were performed by varying different method parameters such as buffer concentration, pH of mobile phase, flow rate, and column temperature. The separation was performed on ProntoSIL C18 column (250 mm × 4.5 mm; 5µm) at room temperature using Acetonitrile: methanol in the ratio of 75:25 (v/v) in isocratic condition at a flow rate of 1.0 mL/min. The detection was performed by an ultraviolet detector (UVD) at 223 nm with total run time of 10 min. Calibration curves were linear in the concentration range of 20-120 µg/mL for with correlation coefficients of 0.9996. LOD and LOQ were found to be 0.040 µg/mL and 0.132 µg/mL proves the sensitivity of the developed method. The method can effectively separate the degradation compounds during the stress study and the standard drug Plazomicin was found to be stable in all the stress degradation conditions. The developed method was able to determine the contents of the Plazomicin commercial dosage forms and hence the method was used for the routine analysis of Plazomicin in bulk drug as well as in pharmaceutical formulations.

KEYWORDS: Plazomicin, HPLC analysis, Method validation, & High-Performance Liquid Chromatography.

INTRODUCTION
Pharmaceutical analysis is traditionally defined as analytical chemistry dealing with drugs both as bulk drug substances and as pharmaceutical products (formulations). However, in academia, as well as in the pharmaceutical industry, other branches of analytical chemistry are also involved, viz. bioanalytical chemistry, drug metabolism studies, and analytical biotechnology.[1, 2] The development of drugs in the pharmaceutical industry is a long-term process, often in all these steps the amount of data generated is enormous.[3] Product analysis involves dealing with the various formulations used for toxicological studies, clinical studies, and marketing.[4] For both substances and formulations there is an increasing interest in the introduction of process analytical chemistry.[5] Biomolecules, i.e., macromolecules such as proteins or hormones, either produced by isolation from biological sources or by means of biotechnology, must also be subjected to careful analytical controlup.[6]

There are a number of regulations that have to be followed in the development of pharmaceuticals as well as in their production. Regulatory approval is required prior to each clinical trial and before marketing is licensed.[7,8] An important part of the development process is safety evaluation, primarily the toxicology tests, which run from 1 to 24 months in different species.[9] Quality is important in every product.[10] The methods of estimation of drugs are divided into physical, chemical, physico-chemical and biological ones.[11,12] The combination of mass spectroscopy with gas chromatography is one of the most powerful tools available.[13] It is not only the moral responsibility of manufacturers to produce effective, safe and non-toxic forms but also their legal responsibility.[14-16] The importance of chromatography is increasing rapidly in

pharmaceutical analysis.[17] HPLC is a special branch of column chromatography in which the mobile phase is forced through the column at high speed.[18]

Plazomicin is an aminoglycoside antibiotic used to treat complicated urinary tract infections. Plazomicin is approved by the U.S. Food and Drug Administration (FDA) for adults with complicated urinary tract infections, including pyelonephritis, caused by Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, or Enterobacter cloacae, in patients who have limited or no alternative treatment options.[19]

Plazomicin has been reported to demonstrate in vitro synergistic activity when combined with daptomycin or ceftobiprole versus methicillin-*resistant Staphylococcus aureus, vancomycin-resistant S. aureus* and against *Pseudomonas aeruginosa* when combined with cefepime, doripenem, imipenem or piperacillin/tazobactam.[20] Plazomicin was found to be noninferior to meropenem. The molecular structure of Plazomicin was shown in figure 1.

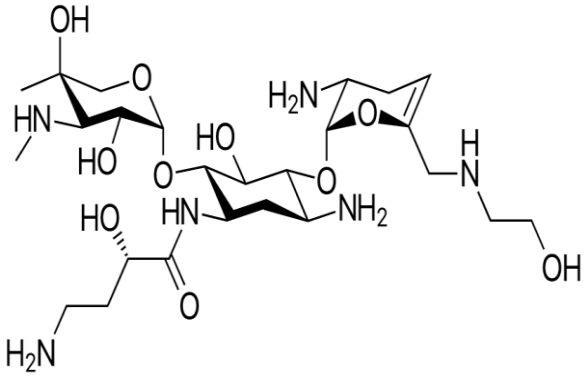

Figure 1: Molecular structure of Siponimod.

Plazomicin exerts a bactericidal action against susceptible bacteria by binding to bacterial 30S ribosomal subunit. Aminoglycosides typically bind to the ribosomal aminoacyl-tRNA site (A-site) and induce a conformational change to further facilitate the binding between the rRNA and the antibiotic. This leads to codon misreading and mistranslation of mRNA during bacterial protein synthesis.[21] Plazomicin demonstrates potency against *Enterobacteriaceae*, including species with multidrug-resistant phenotypes such as carbapenemase-producing bacteria and isolates with resistance to all other aminoglycosides.[22]

The literature survey for the available analytical methods for the analysis of Plazomicin confirms that there are very few analytical methods available for the estimation of Plazomicin and its related impurities in formulations using HPLC[23] and one HPLC bio-analytical method reported for the estimation of plazomicin in human plasma[24] only. No other analytical method available for the assay of Plazomicin and also there is no analytical method reported for the estimation of Plazomicin in pharmaceutical formulations.

The aim of the research work to develop and validated analytical method for the determination Plazomicin in single dosage form and estimation of degradants generated during storage of finished products using techniques such as High-performance liquid chromatography.

MATERIALS AND METHODS

Instrumentation

The details of the instruments used in the study were given in table 1.

Table 1: Instrumentation Details for the present study.

S No	Instrument	Model
1	HPLC system	LC – 7000, PEAK HPLC (India)
2	Pump	LC-P7000
3	Injection mode	Manual
4	Injector	Rheodyne type [model 7725]
5	Injection volume	20 µL fixed volume loop
6	Injection	Hamilton [USA]
7	Detector	UV Detector
8	Software	Autochro -3000 [Young Lin - Korea]
9	Weighing Balance	Denver - SI-234 [Bohemia]
10	UV-Visible spectrophotometer	Teccomp UV-2301 [India]
11	pH meter	Systronics - Sr No S 1326 [India]
12	Ultrasnic Bath Sonicator	GT Sonic [India]
13	Vacuum filtration	Borosilicate vacuum filtration kit
14	Membrane filter (0.2 µ)	Merck Millipore [USA]

Chemicals and solvents

The working standard drug Plazomicin (98.76% purity) along with the formulation injection dosage form (Zemdri® - 500 mg/10mL) were obtained from Cipla Pharmaceuticals, Hyderabad. HPLC grade Methanol, Water and Acetonitrile were purchased form Merk chemicals private limited, Mumbai. The buffer solutions used for the study were AR Grade and purchased from Merck Specialties Private Limited, Mumbai, India.

Preparation of standard drug solution

Preparation of standard stock solution was the primary step prior to experimental work. A standard stock solution of 1000 µg/mL was prepared by weighing accurately 10 mg of the standard drug Capmatinib and was taken in a 10 mL volumetric flask having little amount of Methanol. Dissolve the drug in the solvent and make up to the mark. Then it was filtered through 45µ filter paper to remove un-dissolved particles or any solid substances. By diluting the standard solution with mobile phase, different concentrations (20, 40, 60, 80,100&120µg/mL) of standard solutions was prepared.

Preparation of formulation solution

The injection vials of Zemdri® brand containing 500 mg/10mL of Plazomicin was utilized for the preparation of formulation solution in the study. Then an amount of injection solution equivalent to 25 mg of Plazomicin was accurately measured and taken in a 25 mL volumetric flask containing 10 mL of methanol. The flask was sonicator for 5 min to dissolve the drug completely in solvent and filtered through 0.45 µ membrane filter. The flaks were made up to the mark using same diluent and then it was diluted while doing the formulation analysis.

HPLC Method Development

Selection of wavelength

To select an appropriate monitoring wavelength, the standard solutions of 10 µg /mL was prepared and scanned by the UV-Vis spectrophotometer. The obtained wavelength maxima were selected as suitable wavelength for the detection.

Selection of stationary phase

Since the Plazomicinis a Polar drug, a non-polar C18 column was selected for the separation of the drug. Different columns of different companies, manufactures and configurations were tested.

Method Development trails

Table 02: Method Development trails HPLC method development.

Trail.no	Parameter	Condition	Trail.no	Parameter	Condition
I	MP	Methanol: Water in 50:50 (v/v)	**IV**	MP	methanol: acetonitrile 50:50 (v/v)
	Wavelength	223 nm		Wavelength	223 nm
	Stationary Phase	ProntoSIL C18 column (250mm × 4.5 mm; 5µm)		Stationary Phase	ProntoSIL C18 column (250mm × 4.5 mm; 5µm)
	Flow Rate	1.0 mL/min		pH of MP	5.2
				Flow Rate	1.0 mL/min
II	MP	Water: Methanol in 75:25 (v/v)	**V**	MP	Methanol: acetonitrile in 75:25 (v/v)
	Wavelength	223 nm		Wavelength	223 nm
	Stationary Phase	ProntoSIL C18 column (250mm × 4.5 mm; 5µm)		Stationary Phase	ProntoSIL C18 column (250mm × 4.5 mm; 5µm)
	Flow Rate	1.0 mL/min		pH of MP	5.2
				Flow Rate	1.0 ml/min
III	MP	Water: Methanol in 25:75 (v/v)	**VI**	MP	Methanol: Acetonitrile: Water 60:20:20 (v/v)
	Wavelength	223 nm		Wavelength	251 nm
	Stationary Phase	ProntoSIL C18 column (250mm × 4.5 mm; 5µm)		Stationary Phase	Spherisorb ODS C18 Column (250 x 4.6 mm and 5µm)
	pH of MP	5.2		pH of MP	5.1
	Flow Rate	1.0 mL/min		Flow Rate	1.0 mL/min
				Pump Mode	Isocratic

Analytical Method Validation

The method was validated with respect to linearity, accuracy, precision, repeatability, selectivity, and specificity, according to the ICH guidelines. Validation studies were carried out by replicate injections of the sample and standard solutions into the chromatograph.

Specificity

Specificity of the method was checked by injecting the solution into the chromatograph. Specificity of the method was assessed by comparing the chromatogram of Plazomicin (standard), blank and sample solutions to those obtained for tablet solutions. Retention time of the Plazomicin in standard solution, and in the sample solution was compared to determine the specificity of the method.

System suitability

The system suitability was determined by making six replicate injections of the standard solution and analyzing Plazomicin for its peak area, peak USP tailing factor, and number of theoretical plates. The proposed accepted criteria are not more than 2% for RSD%, not less than 2 for resolution, not more than 2 for USP tailing

factor, and not less than 2000 for the number of theoretical plates.

Sensitivity of the method

The limit of detection (LOD) and limit of quantitation (LOQ) were defined as the lowest concentration of analyte in a sample that can be detected and quantified. The standard solutions of Plazomicin for LOD and LOQ were prepared by diluting them with suitable solvent. The LOD and LOQ were determined by the signal-to-noise (S/N) ratio for each compound through analyzing a series of diluted solutions until the S/N ratio yield 3 for LOD and 10 for LOQ, respectively.

Linearity and Range

The calibration curve in the developed method was constructed from LOQ concentration. Plazomicin standard stock solution of 1 mg/mL was used for preparation of subsequent aliquots. Sample solution was loaded and 20 µL was injected into column. All measurements were repeated for each concentration. The calibration curve of the area under curve versus concentration was recorded. Form the calibration curve, correlation and regression values were calculated for Plazomicin.

Precision

The precision studies were carried out by estimating response of Plazomicin six times at a standard concentration of 80 µg/mL and results are reported in terms of %RSD. The intra-day and inter-day precision studies were carried out by estimating the corresponding responses six times on same day for intraday and interday for three different days and it was expressed as the percentage relative standard deviation (%RSD) which was calculated as per the following expression

%RSD = (standard deviation / mean) x 100.

Accuracy/ Recovery

Accuracy of method was observed by recovery result from two placebos preparations accurately spiked with different concentration of Plazomicin. Recovery assessment was obtained by using standard addition technique which was by adding known quantities of pure standards at three different levels in 50%, 100% and 150% to the pre analyzed sample formulation. From the amount of drug found, amount of drug recovered and percentage recovery were calculated by using the formula.

%RSD = (standard deviation / mean) x 100.

Ruggedness

Two laboratory analysts carried out the precision of Plazomicin at a standard concentration of 80 µg/mL was prepared by different analysts in the laboratory conditions, the prepared solution were analyzed in the optimized conditions. Peak area that obtained was used for the determination of ruggedness of the method. Ruggedness was expressed in terms of %RSD which must be less than 2.

Robustness

Robustness of the proposed method included six deliberate variations to some chromatographic parameters. The modifications include different mobile phase ratios and different detector wavelengths and different percentage in the mobile phase (in the range of ± 5 of the nominal value and the normal %). The % change in each of the changed condition was calculated.

Formulation analysis

This proposed method was applied to the determination of Plazomicin in commercially combined tablets. The sample solution at a concentration of 80 µg/mL of Plazomicin was analyzed in the optimized conditions. Peak area of the resultant chromatogram was used for the estimation of assay using label clime recovery method. The % assay was calculated for Plazomicin using the standard calibration values.

Forced degradation study

Table 3: Methodology for forced degradation study.

S. No.	Degradation type	Experimental conditions	Time
1	Acid Hydrolysis	50 mg of drugs were mixed with 50 mL of 0.1N HCl solution. The solution was neutralized, diluted up to standard concentration (80 µg/mL) and was analyzed in the developed method condition	24 Hours
2	Base Hydrolysis	50 mg of drugs were mixed with 50 mL of 0.1N NaOH solution. The solution was neutralized and diluted up to standard concentration i.e 80 µg/mL and was analyzed in the developed method condition	24 Hours
3	Oxidative Degradation	50 mg of drugs were with 50ml of 3% Peroxide solution. The solution was neutralized and diluted up to standard concentration (80 µg/mL) and was analyzed in the developed method condition	24 Hours
4	Photolytic Degradation	50 mg of drug sample was kept in UV light [254 nm]. After the selected time of light expose, the drug solution was prepared and was analyzed	24 Hours
5	Thermal Degradation	50 mg of drug sample was kept in oven at 60 ^{0}C. After the selected time of light expose, the drug solution was prepared and was analyzed	24 Hours

RESULTS AND DISCUSSION

The present work aimed to develop a simple and accurate HPLC method for the quantification of Plazomicin in pharmaceutical formulations. To develop a precise, accurate and suitable RP- HPLC method for the simultaneous estimation of Plazomicin in different mobile phases were tried and the proposed chromatographic conditions were found to be appropriate for the quantitative determination. Proper selection of the stationary phase depends up on the nature of the sample, and molecule's physico- chemical properties. The ultraviolet absorption spectra of the Plazomicin demonstrated that the maximum absorption at a wavelength near 223 nm, and it was therefore chosen during the entire study.

Mixture of acetonitrile and methanol in the ratio of 75:25 (v/v) was selected as mobile phase and the effect of composition of mobile phase on the retention time of Plazomicin was thoroughly investigated. Proper selection of the stationary phase depends up on the nature of the sample, molecular weight and solubility. The drug Plazomicin is non - polar. Non-polar compounds preferably analyzed by reverse phase columns. Between C_8 and C_{18}, C_{18} column was selected. Non-polar compound is very attractive with reverse phase columns. So the elution of the compound from the column was influenced by polar mobile phase. The system suitability results obtained for proposed method were within acceptable limits (capacity factor >2.0, tailing factor =2.0 and theoretical plates >2000), thus, the system meets suitable criteria.

In the trail6, single sharp symmetric peak with acceptable system suitability was observed (Figure 2).

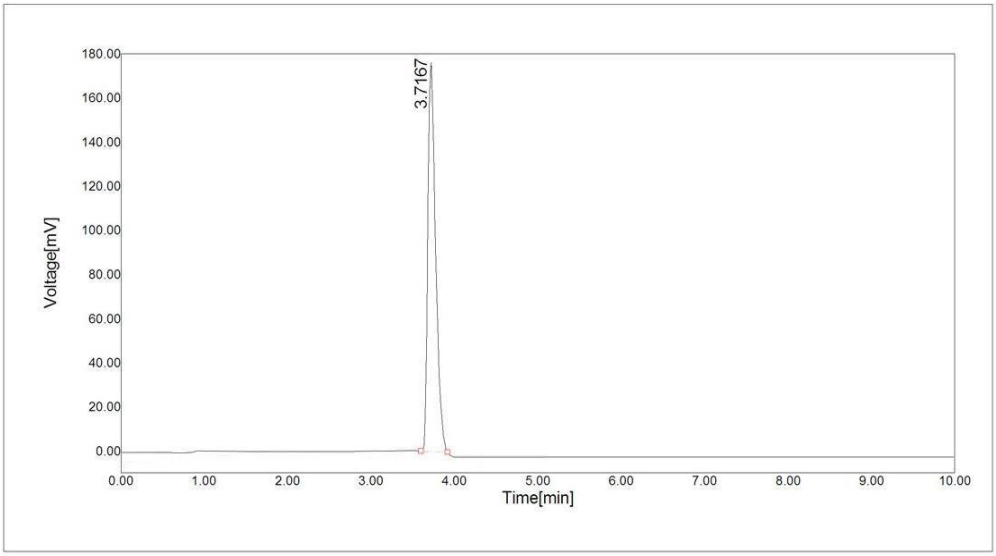

Figure 2: Optimized Chromatogram observed for Plazomicin.

Hence these conditions were found to be suitable and further valuation was carryout using these conditions. In the optimized conditions, well resolved, retained and accepted system suitability was observed. The optimized conditions were given in table 4.

Table 4: Optimized chromatographic conditions.

S. No	Parameter	Results
1	Mobile phase	Acetonitrile: methanol in the ratio of 75:25 (v/v)
2	Wavelength	223 nm
3	Stationary Phase	ProntoSIL C18 column (250mm × 4.5 mm; 5µm)
4	pH of MP	5.3
5	Flow Rate	1.0 mL/min
6	Pump Mode	Isocratic
7	Pump Pressure	9.1±4 MPa
8	Run time	10 min

The calibration curve for Plazomicin was obtained by plotting the peak area of Plazomicin versus concentration of Plazomicin over the range of 20-120 µg/mL, and it was found to be linear with r = 0.999. The regression equation for Plazomicin was found to be y = 8240.7x - 7207.3 (R^2 = 0.9996). Precision was evaluated by carrying out six independent sample preparation of a single lot of formulation.

Table 5: Precision results.

S. No	Intraday Precision	Interday Precision
1	645129.1	646741.9
2	649251.5	651523.9
3	643281.7	644632.6
4	645258.5	646032.8
5	644151.9	645762.3
6	645213.9	647343.1
% RSD	**0.32**	**0.37**

Percentage relative standard deviation (% RSD) was found to be less than 2% for within a day (Intra-0.32 %), day to day (Inter-0.37 %) variations and ruggedness (0.48 %), which proves that method is precise.

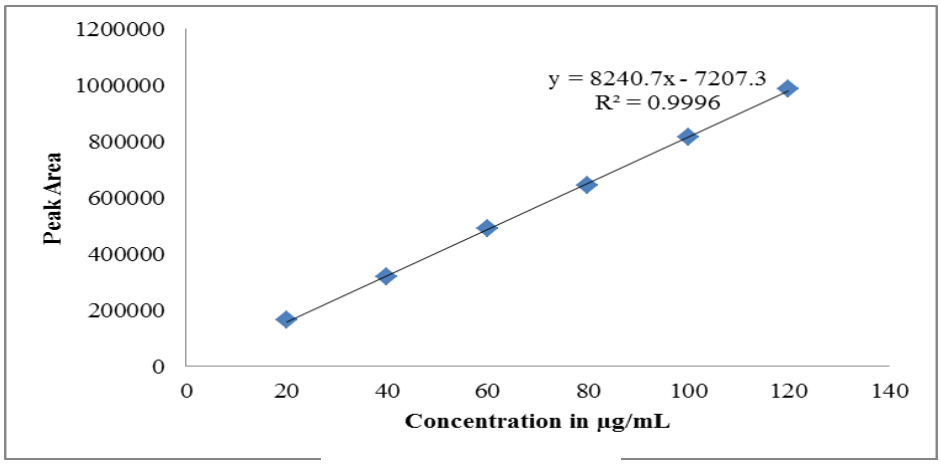

Figure 3: Linearity graph.

To check the degree of accuracy of the method, recovery studies were performed in triplicate by standard addition method at 50%, 100% and 150%. Results of recovery studies are shown range 98.59 - 99.81%. The mean recovery data obtained for each level as well as for all levels combined were within 2.0% of the label claim for the active substance with an R.S.D. < 2.0%, which satisfied the acceptance criteria set for the study.

Table 6: Forced degradation study results.

S No	Condition studied	No of degradation compounds separated	% assay	% degradation
1	Acid	4`	94.01	5.99
2	Base	3	95.25	4.75
3	Peroxide	2	95.32	4.68
4	Thermal	1	96.85	3.15
5	UV light	2	94.58	5.42

To evaluate the robustness of the developed RP-HPLC method, small deliberate variations in the optimized method parameters were done. The effect of change in flow rate, pH and mobile phase ratio on the Area were studied. These parameters were found to change proportionally.

When stress conditions were applied to Plazomicin, the HPLC results showed that there was no interference between the tested drug and the degradation products. Peak purity results were also within the acceptable limit for all the degradation conditions studies confirms that the Plazomicin peak is homogeneous in all stress conditions. In all the stress degradation conditions, the standard drug Plazomicin was effectively separated, identified and quantified. The % assay of Plazomicin was found to be very high and the % degradation was found to be very less in the developed method. The degradation products were found to be 4, 3, 2, 1 and 2 in acidic, base, peroxide, thermal and UV light conditions respectively. Among the degradation conditions studied, very high % degradation was observed in acid condition in which the % degradation was observed to be 5.99 % with 4 additional degradation products. Very less % degradation was observed in thermal condition in which the % degradation of 3.15 % obtained confirms that the drug was more stable in thermal conditions. All the degradation products were effectively separated and there is no overlap of degradation compounds with the standard drug. Hence the developed method was found to be stability indicating.

The developed method was applied for the estimation of Plazomicin in its formulation solution prepared from

Zemdri® brand tablets of Plazomicin. The % assay in formulation analysis was found to be 99.60 for Plazomicin in the developed method. More than 98% assay was observed in the developed method. Hence the method was found to be suitable for the routine analysis of Plazomicin in bulk drug as well as formulations.

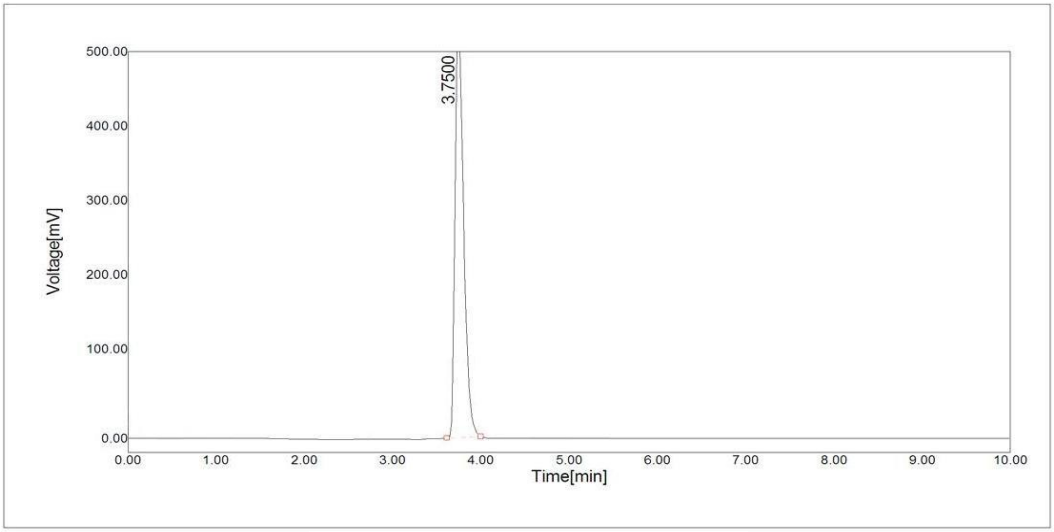

Figure 4: Formulation chromatogram.

Table 6.9: Formulation results.

S. No	Drug	Brand	Label claim	Concentration prepared	Concentration found	% assay
1	Plazomicin	Zemdri®	500 mg/10mL	81 µg/mL	79.682 µg/mL	99.60

The sensitivity test results of Plazomicin indicated that the method was sensitive enough to detect a concentration of 0.040 µg/mL and able to quantify at a concentration of above 0.132 µg/ml. The proposed method uses a simple mobile phase composition which is easy to prepare. The rapid run time of 10 min and the relatively low flow rate allows the analysis of large number of samples with less mobile phase that proves to be cost-effective. Efficient UV detection at 223 nm was found to be suitable without any interference from injectable solution excipients or solvents. The method was validated showing satisfactory data for all the method validation parameters tested. The developed methods can be conveniently used by quality control laboratories. The summary results observed method development, validation and application of the analytical method developed for the analysis of Plazomicin in formulations was given in table 7.1.

Table 7: Summery results achieved in the method developed for analysis of Plazomicin.

Study	Parameter	Results
Method Developed	Elution	Isocratic
	Mobile Phase	Acetonitrile: methanol in the ratio of 75:25 (v/v)
	pH	5.3
	Column	ProntoSIL C18 column (250mm × 4.5 mm; 5µm)
	Wavelength	223 nm
	Flow	1.0 mL/min
	Runtime	10 min
	Temperature	Ambient
Method validation	Retention Time	3.7 min
	Theoretical Plates	7815
	Tailing Factor	1.09
	Resolution	--
	Linearity range	20 to 120 µg/mL
	Slope	8240.7
	Intercept:	- 7207.3
	r^2 (correlation coefficient)	0.9996
	Intraday Precision	0.32
	Interday Precision	0.37

	Ruggedness	0.48
	Recovery	98.59 - 99.81
	% change in Robustness	0.48 to 1.60
	LOD	0.040 µg/mL
	LOQ	0.132 µg/mL
Method Application	% Degradation in Acidic Base Peroxide Thermal UV Light	 5.99 4.75 4.68 3.15 5.42
	Formulation assay	99.60

CONCLUSION

A simple, highly sensitive, isocratic stability indicating reversed phase-high performance liquid chromatography (RP-HPLC) method with UV detection at 223 nm was developed and validated for analysis of Plazomicin. Retention time of the Plazomicin was found to be 3.7 min. A mobile phase consisting of acetonitrile and methanol in the ratio of 75:25 (v/v) at flow rate of l.0 mL/min was employed in this study. The calibration curves were linear in the concentration range of 20 to 120 µg/mL with regression coefficient (r^2) of 0.9996. The limits of detection (LOD) and the limits of quantification (LOQ) were found to be 0.040 and 0.132 µg/mL, respectively. The method was statistically validated in accordance with international conference on harmonization (ICH) guidelines. The method can effectively separate the stress degradation compounds formed during the stress study and the % drug content was observed to be very high in all the stress studies. Hence based on the statistical analysis of the data it has been unequivocally construed that the method is reproducible and selective for the routine analysis of Plazomicin in bulk drug and tablet dosage form.

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