A VALIDATED BIO ANALYTICAL RP-HPLC METHOD FOR ESTIMATION OF PLAZIMYCIN IN HUMAN PLASMA WITH INTERNAL STANDARD

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Abstract:

A reliable, simple, sensitive, stable, bioanalytical RP-HPLC (Reverse phase High Performance liquid chromatography) for determination and estimation PZM in human plasma was developed and validated with NFP as internal standard. The drug PZM was spiked with plasma and liquid - liquid extraction was carried out by using acetonitrile and methanol. The chromatographic separation was performed with Knauer C18 vertex plus column with 250 X 4.6mm dimensions and packing material Eurospher II 100-5. The isocratic elution of methanol, Acetonitrile and 1.0% triethylamine in the ratio of 30:35:35 (v/v) with ultraviolet detector at 214. The flow rate of the isocratic pump was set to be 1 mL/min. The developed method was validated as per the guidelines of ICH and linear calibration curve was found linear over the range of 50-450 ng/mL concentration with regression equation of y = 0.0035x + 0.1381 with correlation coefficient 0.995 (R^2) obtained was satisfactory**.** The validation results of the method was found accurate (percent recovery 95-105), precise (<2.0%) and stable. Thus the developed RP-HPLC can be used for regular invitro and invivi estimation of PZM**.**

Keywords: Bioanalytical, RP-HPLC, Plazomicin(PZM), Validation, ICH guidelines.

Introduction:

Plazomicin (PZM) drug is an aminoglycoside antibiotic used for the treatment of complicated urinary tract infections [1]. The drug is approved in 2018 by US FDA (Unites states Food and drug adminstration) and listed on the WHO's (World health organization) essential medicines.

[2]. The drug indicated for adult patients for complicated urinary tract infections (c UTI) including pyelonephritis which is caused by *Escherichia coli, Klebsiella pneumonia, Roteus mirabilis* and *Enterobacter clocae. The* antibacterial activity of the PZM is mediated by binding of the drug to bacterial 30S ribosomal sub unit and inhibits the protein synthesis label. It is also reported to demonstrate invitro synergistic activity when it is combined with other drugs kije Dapomycin or Ceftobiprole versus methicillin resistant staphylococcu aureus, Vancomycin resistant *staphylococcus aureus and against Pseudomona aeruginosa* when combined with Cefepime, Doripenem, Imipenem or or Poperacillin/Tazobactuam [3]. The drug also exhibits potent in vitro activity versus Carbapenem resistant *Acinetobacter baumannii* [4]. PZM as available as injection in to vein and it works by decreasing the ability of bacteria to make protein. General side of the usage of PZM includes kidney problems, nausea, diarrhea and blood pressure changes. Severe side effects with usage include hearing loss, clostridium difficult assaociated diarrhea, anaphylaxis and muscle weakness. Usage of PZM during the pregnancy may harm the baby.

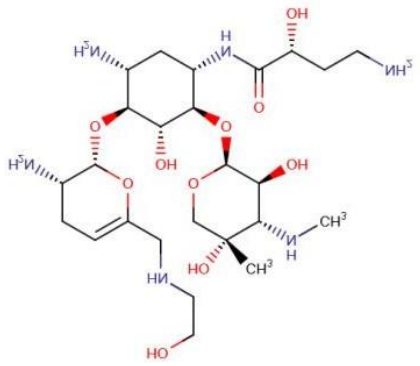

Figure 1: Chemical structure of Plazomicin (PZM)

The present work is aimed to develop a bioanalytical liquid chromatography method for estimation of PZM in plasma. The pharmaceutical field requires the analytical investigation of bulk drugs, intermediates, drug products, formulations, impurities, degradation products and biological samples containing drugs and their metabolites. The intention of the development of bio analytical methods for human clinical pharmacology, bioavailability (BA) and bioequivalence (BE) requiring pharmacokinetic(PK) evaluation including human pharmacology, toxicology studies preclinical studies to analyze the drug in biological articles such as blood

plasma urine and serum [5]. There are plenty of instrumental and chemical methods have been developed at regular intervals which are involved in the estimation of drugs to serve their purposes. Among other analytical techniques, HPLC is the most utilized diagnostic device as a part of medication investigation. There is only one HPLC method was described for analysis of PZM and impurities [6]. As there is no bioanalytical HPLC methods have not been reported with PZM drug, the present work is going to be novel method for analysis of drug in biological matrices. The drug Nefidipine (NFP) is used an internal standard which is a first generation dihydropyridine L-type calcium channel blocker, used for treatment of Angina and hypertension.

Experimental:

Chromatography instrument:

The experiment was carried out with the Agilent 1100 series HPLC system with quaternary G1311 A pump, COCCOM G1316A thermostat column temperature control, Thermostatic autosampler G 1329 A with sample volume of 0.1-1500 µL and G 1314 A variable programmable UV detector. The Agilent 1100 chromatographic system is operated and integrated with the Agilent chem. station software. The separation was carried with the Knauer C 18 chromatographic column with 250 X 4.6mm dimensions and packing material Eurospher II 100-5.

Chemicals and Solvents:

Solvents like Acetonitrile, Methanol and water of HPLC grade were purchased from the Merck chemicals private limited, Mumbai. AR grade triethylamine, dichloromethane, methanol, acetonitrile, chloroform and diethyl ether were purchased from Merck chemicals private limited, Mumbai. Standard drugs of PZM and NFP were procured from reputed laboratories.

Preparation of mobile phase:

The mobile phase was prepared by mixing the Methanol, Acetonitrile and 1.0% triethylamine in the ratio of 30:35:35 (v/v) in to mobile phase container and sonicated for fifteen minutes to ensure the homogeneous mixing using ultrasonicater. Then the prepared mobile phase was, and then filtered through 0.45 µ nylon membrane filter paper using vacuum filtration set.

Preparation of primary stock solution of PZM:

Primary stock solution of PZM and NFP prepared individually at 1mg/ml in acetonitrile. An accurately weighed 10mg PZM was dissolved in 10ml of acetonitrile to prepare 1mg/mL of standard stock solution was stored in the Stoppard volumetric flask and kept at 4^0C, prior to HPLC analysis.

Preparation of NFP internal standard (IS) stock stock and working solutions:

10mg of NFP standard was dissolved in 10 mL of acetonitrile to prepare 1mg/mL of standard stock solution in stoppared volumetric flask. The NFP internal standard stock solution was further diluted with acetonitile solution to prepare 1µg/mL of NFP working solution.

Preparation of Sample solution:

The human blood was collected from local blood bank and plasma was collected by centrfugaton at 400 rpm for 15 minutes at 4^0C. Stock solutions of of PZM and NFP were prepared and spiked in to plasma to prepare plasma standards of both drugs. Various solvents have been evaluated including dichloromethane, methanol, acetonitrile, chloroform and diethyl ether for the extraction of drug from plasma. The drug extraction process was conducted by adding the 100µL of plasma to the 50 µL of standard stock solution and mixed for 1 miunte in vortex mixture. A mixture of methanol and acetonitrile (50:50 v/v) solution was added to the plasma for extraction of drug from the biological matrix. The liquid –liquid extraction was carried for vortex of the mixture solution for 30seconds and centrifuged at 4°C at 5000rpm. The same procedure of repeated with the 1mL of plasma without the drug in order to prepare the blank plasma solution. The supernatant was collected, filtered and used as a blank solution for analysis.

Preparation of calibration solutions:

Preparation of calibration curve (CC) standards and internal standard solutions by dissolving the stock solution (1000 µg/mL) of PZM drug standard and NFP with methanol and final concentrations ranging from 50-450 ng/mL. An aliquots of 0.9 mL of the blank plasma was spiked with 0.1 of working standard solution to prepare the calibration concentrations containing 50, 100, 150, 200, 250, 300, 400 and 450 ng/mL of PZM respectively. Similarly NFP internal standard solutions are also prepared at constant concentration of 1 µg/mL. The validation of the method was conducted at three levels of CC concentrations i.e 100 ng/Ml of low quality control

(LQC), 300ng/mL of middle quality control and 450ng/mL of high quality control (HQC) respectively.

Method development and optimization:

Initially the method conditions limited and optimized to columns, mobile phase, and detector wavelength. As the drug is polar compound, different polar solvents with different ratio were tested in order to separate and identify the drug and its internal standard. Various columns columns ranging from C8 to C18 were tested with varying the different combinations of mobile phase solvents. Among studied trials isocratic elution of methanol, Acetonitrile and 1.0% triethylamine in the ratio of 30:35:35 9v/v) with C18 column found suitable condition for separation and identification PZM and NFP. Ultraviolet detector of HPLC was found at 214 optimum wavelengths for determination of both drugs. Optimization of method conditions like change in pH and flow rate of the mobile phase solvent, injection volume, column temperature etc also conducted in order to achieve the better separation and area integrations from the chromatograms.

Method validation:

The proposed method was validated for linearity, precision, recovery, ruggedness and robustness according to the ICH guidelines and US FDA guidelines [7-9]. All the validation parameters were conducted at three levels of concentration ranges i.e LQC (100 ng/mL, MQC (300 ng/mL and HQC (450 ng/mL). The proposed HPLC method selectivity was evaluated by analyzing the blank plasma samples for endogenous interference or matrix effect of the plasma component. The linearity was evaluated by injecting the various concentrations of the PZM drug along with internal standard solution and the linear calibration with high sensitivity wwas selected as calibration range for validation. The recovery of extraction PZM from the biological matrix was evaluated by comparing the concentrations measured in the plasma with concentration added in aqueous solution. The LLOD (lower limit of detection) was with proposed method was calculated from the calibration curve. The solution stability study [10] was conducted at LQC (100 ng/mL, MQC (300 ng/mL and HQC (450 ng/mL) cocnetration to determine the storage period and it's correlated with the initial concentration as zero cycle. Freeze–thaw stability was determined by frozen plasma samples were thawed at room temperature for 2 hours and frozen for 24 hours prior analyzed and compared the results with that of zero cycle. The short term

stability (Bench top stability) of the method was studied by samples kept at room temperature for 24 hours. After that the samples were analyzed and the results were compared with that of the zero cycle.

Result and discussion:

Method development:

The goal of developing a bioanalytical assay method condition for estimation of PZM by RP-HPLC**.** After several trials for development of bioanalytical method mixture of methanol, acetonitrile and 1.0% triethylamine in the ratio of 30:35:35 (v/v) was shown high peak performance like average peak height, resolution and symmetry. Triethylamine was used to basify the mobile phase and improved peak symmetry. Separation was carried out using Knauer C18 vertex plus column with 250 X 4.6mm dimensions and packing material Eurospher II 100-5 at column temperature of 35^0C. The pH of the optimized mobile phase was found to be 9.5 and pumped at mobile phase of 1 ml/min. The volume for injection was kept at 20 µL for the sample analysis and detector wavelength was fixed at 214nm. Compared to a standard solution, the matrix effect can cause response of the different compounds present in the biological matrix. The important issue of the biological method development is the possibility of matrix effect nu suppression or enhancement of the standard response. The black chromatogram of the plasma sample without drug under proposed optimized conditions was studied to to evaluate the selectivity of the proposed method. The chromatogram showed noise of the base from endogenous substances but no interference was observed at retention time of PZM at 5.68min and NFP at 3.76min. Typical chromatograms of blank sample, standard soluble with acetonitrile and plasma spiked standard drugs were presented in figure 2, 3 and 4 respectively. No carryover effects were observed when blank sample solution was analyzed after HQC sample injections.

Linearity, Precision and Recovery:

Linearity was performed at using standard analytical curves and showed good linearity between ratio (of response of standard and IS (internal standard) and the corresponding concentration of the PZM standard drug over a concentration range of 20-450 ng/ml. The calibration curve obtained was $y = 0.0035 x + 0.1381$ with correlation coefficient 0.995 (R^2) obtained was satisfactory. As per ICH guidelines, LLOQ is described as the lowest concentration of the analyte in sample that can quantified with precision and accuracy (RSD < 15%) and LLOQ (50

ng/ml) of the PZM was obtained for the lowest concentration of calibration curve [14]. The LLOQ is use to quantity the low concentration of the PZM biological matrix. Hence the method shows good sensitivity for analysis of PZM. Precision (intraday & interday) precision and accuracy (Recovery) of the proposed method was evaluated by using three QC samples at the concentration range from ng/ml.

The result of the precision and accuracy were obtained in the presence of biological matrix were analyzed at concentration range of 100 ng/Ml (lowest concentration), 300 ng/Ml (middle concentration) and 450 ng/mL (high concentration). The RSD (Relative standard deviation of the six analytical run values of the intraday and interday obtained for PZM. The RSD of the intraday precision was found 1.07, 1.04 and 1.78 at HQC, MQC and LQC respectively. The RSD of the interday precision was found 1.90, 1.21 and 1.46 at HQC, MQC and LQC respectively. Precision results were presented in the table 3 and 4. The within (intra) and between (inter)day precision RSD % values were below 2% at all QC levels.

The QC samples recoveries over the sample preparation process were calculated as the % of the PZM peak area from the processed QC samples and unprocessed QC standard solutions acetonitrile. The result of recovery studies at three HQC, MQC and LQC were presented in table 5. These results proved that change all the assay results were not more than 5% and also the relevance of using standard solutions in acetonitrile to prepare the calibration curve. Therefore, the bioanalytical RP-HPLC method can be considered precise and accuracies. With LOQ of 100ng/ml, the proposed method was found sensitive enough and can successfully apply to the intracellular PZM level determination.

In the present study stability of the PZM was studied against Freeze-thaw, short term and long-term stability (bench-top stability) conditions. Stability testing can help to enlighten the behavior of the product in qualitative and quantitative analysis [23]. The stability studies with PZM in acetonitrile were evaluated on HQC, MQC and LQC samples. The results of the stability studies were presented in table 6. No significant changes were observed after exposure of the PZM in different stability conditions. In contrast, the assay results and % of change in the peak area and area ratio revelas that the PZM drug was more stable at short term and long term stability than

the freeze thaw stability where the% or assay was found more that than 5%. The accuracy of the samples found to be nearly equivalent to 100% and RSD value (n=6) of the after stability conditions were found to be less than 15%. The results of the stability studies proves the that there would no issues while short and long term storage of the samples for quantitative studies.

Conclusion:

A Novel RP-HPLC bio analytical method was developed for determination and estimation of the PZM drug in spiked human plasma. The method was successfully separated the standard drug and internals standard. The method was validated as per the ICH guidelines and results of all various parameter were found within acceptable limits. The proposed RP-HPLC method demonstrated excellent stable, accuracy, linearity with sensitivity. The proposed method also has advantage of being rapid and precise, so that it can be applied to the quantification of intracellular PZM at various times of treatment.

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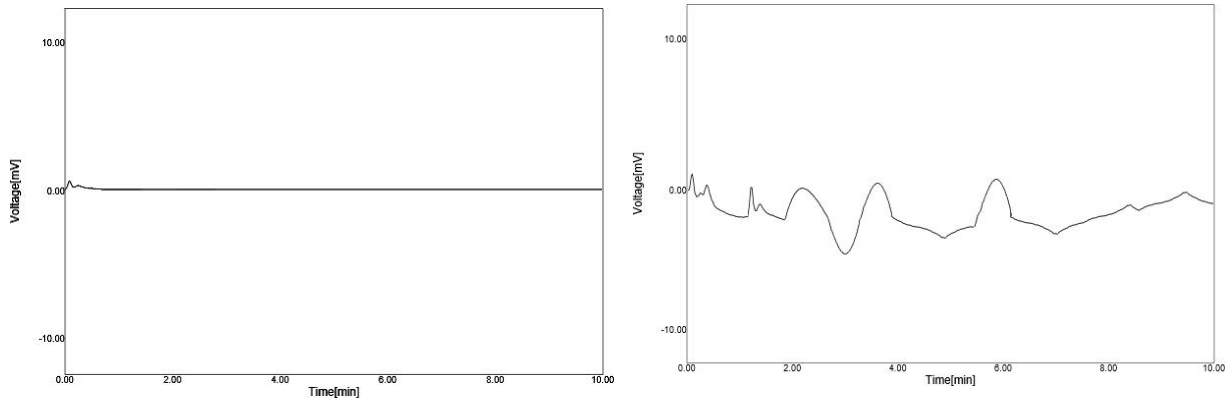

Figure 2: Blank chromatograms of the diluents and spiked plasma sample

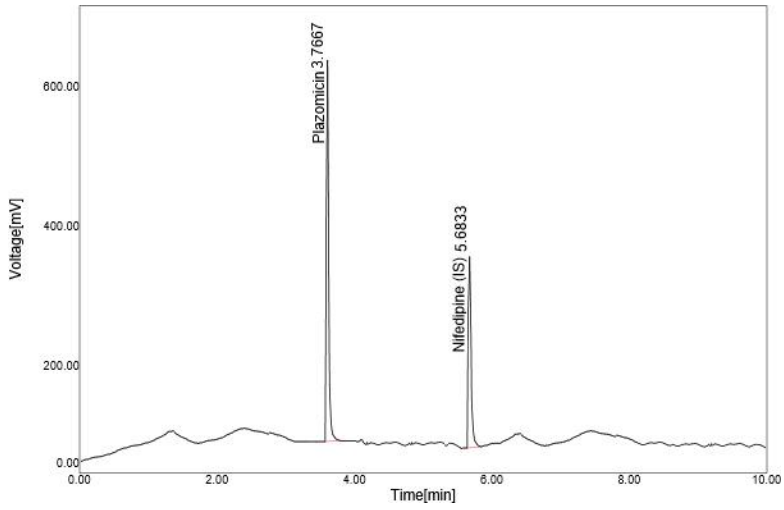

Figure 3: Chromatogram of plasma spiked standard solution of PZM and NFP

Table 1: Linearity of the Plazomicin (PZM) area ratio vs concentration with NFP internal standard

S.No	Concentration in ng/ml	Area of Plazomicin (PZM)	Area of internal standard (NFP)	Ratio
1.	50	20115.13	63211.56	0.318
2.	100	28415.23	62335.32	0.455
3.	150	40215.36	62981.66	0.638
4.	200	53045.84	63012.15	0.841
5.	250	66127.57	62477.15	1.058
6.	300	78845.61	63147.48	1.248
7.	350	81746.21	61178.55	1.336
8.	400	93771.02	62048.47	1.511
9.	450	105314.67	62045.81	1.697
	Correlation coefficient (R^2) = 0.995 **Slope = 0.0035** **Intercept = 0.1381**			

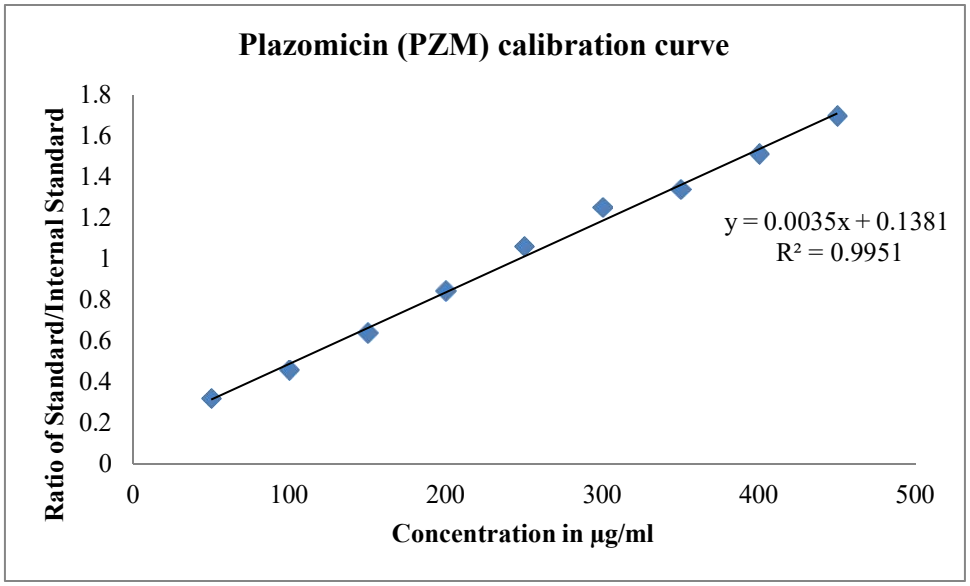

Figure 4: Calibration curve of Plazomicin (PZM) area ratio vs concentration with NFP internal standard

Table 2: Results of Intraday precision:

S.NO	Area ratio at HQC	Area ratio at MQC	Area ratio at LQC
1.	1.63	1.24	0.42
2.	1.66	1.23	0.43
3.	1.65	1.23	0.43
4.	1.64	1.23	0.44
5.	1.61	1.25	0.44
6.	1.64	1.26	0.44
RSD in %	**1.067**	**1.028**	**1.779**

Table 3: Results of Interday precision:

S.NO	Area ratio at HQC	Area ratio at MQC	Area ratio at LQC
1.	1.63	1.25	0.45
2.	1.65	1.23	0.44
3.	1.64	1.23	0.43
4.	1.64	1.24	0.44
5.	1.72	1.24	0.45
6.	1.64	1.21	0.44
RSD in %	**1.903**	**1.210**	**1.465**

Table 4: Results of recovery at HQC:

S.NO	Standard solution in acetonitrile	Plasma spiked	Ratio	% Assay
1.	101374.36	105377.23	1.643	103.94
2.	105547.26	102477.26	1.61	97.09
3.	103125.36	103667.28	1.63	100.52
4.	103784.84	104101.37	1.66	100.30
5.	99122.90	104009.47	1.64	104.93
6.	104129.50	103119.54	1.59	99.03

Table 5: Results of recovery at MQC:

S.NO	Standard solution in acetonitrile	Plasma spiked	Ratio	% Assay
1.	76455.88	77895.02	0.34	101.88
2.	79114.20	77994.61	0.34	98.59
3.	79449.66	78994.33	0.34	99.45
4.	80112.40	79179.22	0.35	98.83
5.	76415.36	79945.56	0.35	104.62
6.	81022.04	77933.18	0.34	96.19

Table 6: Results of recovery at LQC:

S.NO	Standard solution in acetonitrile	Plasma spiked	Ratio	% Assay
1.	30443.26	28991.34	0.13	95.23
2.	27997.03	28831.66	0.12	102.98
3.	28005.51	28443.10	0.12	101.56
4.	30110.57	29415.33	0.13	97.69
5.	28128.59	28615.67	0.12	101.73
6.	28764.23	28569.47	0.12	99.32

Table 6: Results of Freeze thaw stability study

S.NO	Area ratio at HQC	Area ratio at MQC	Area ratio at LQC
1.	1.64	1.23	0.44
2.	1.61	1.22	0.43
3.	1.65	1.25	0.45
4.	1.61	1.25	0.44
5.	1.67	1.23	0.43
6.	1.66	1.26	0.44
RSD in %	**1.533**	**1.17**	**1.722**

Table 7: Results of Short term stability study

S.NO	Area ratio at HQC	Area ratio at MQC	Area ratio at LQC
1.	1.65	1.23	0.45
2.	1.61	1.23	0.44
3.	1.65	1.25	0.45
4.	1.61	1.24	0.43
5.	1.66	1.25	0.44
6.	1.69	1.22	0.44
RSD in %	**1.894**	**0.883**	**1.849**

Table 8: Results of Long term stability study

S.NO	Area ratio at HQC	Area ratio at MQC	Area ratio at LQC
1.	1.62	1.23	0.45
2.	1.63	1.26	0.44
3.	1.65	1.23	0.44
4.	1.65	1.25	0.45
5.	1.62	1.22	0.44
6.	1.61	1.25	0.43
RSD in %	**1.105**	**1.127**	**1.877**