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VALIDATED STABILITY INDICATING MICROBIAL AGAR ASSAY METHOD FOR DETERMINATION OF PRULIFLOXACIN

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ABSTRACT

The development of a specific agar diffusion bioassay for determination of Prulifloxacin is been carried out using the strain *Salmonella typhimurium* ATCC 23564 as a test organism. The validation of the method showed good linearity, precision and accuracy. Drug sample exposed to UV radiation (254nm), acid hydrolysis, alkaline hydrolysis, thermal stress were used to determine the specificity of the bioassay. This study demonstrates the validity of proposed bioassay, which will allow reliable quantification of prulifloxacin in dosage form and can be used as an alternative method for prulifloxacin analysis in stability studies.

Keywords: Prulifloxacin, agar diffusion bioassay, *Salmonella typhimurium* ATCC 23564, validation, stability.

INTRODUCTION

Prulifloxacin is a new flouroquinolone with broad spectrum activity against gram positive and gram negative bacteria[1,2] . It is a prodrug which metabolise in the body to the active compound ulifloxacin. It is been approved for the treatment of uncomplicated and complicated urinary tract infections, community-acquired respiratory tract infections and gastroenteritis, including infectious diarrhoeas. The chemical name of Prulifloxacin is (*RS*)-6-Fluoro-1-methyl-7-[4-(5-methyl-2-oxo-1,3-dioxolen-4-yl)methyl-1-piperazinyl]-4-oxo-4*H*-[1,3]thiazeto[3,2-*a*]quinoline-3-carboxylic acid [3] as shown in chemical structure of Prulifloxacin in figure1.

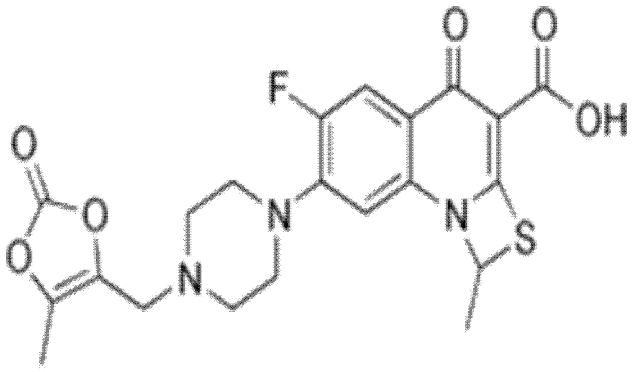

Fig. 1. Chemical structure of Prulifloxacin.

Literature survey revealed some UV-Visible spectrophotometric [4-7], LC-MS/MS [8], RP-HPLC [9],HPLC [10-11]methods. But there were no stability indicating agar diffusion assay methods for the drug Prulifloxacin and hence the main aim of the study was to develop and validate a stability indicating microbial assay for determination of prulifloxacin in commercially available dosage form.

MATERIAL AND METHOD

Chemicals

All the reagents used for the analysis were of analytical grade. Standard Prulifloxacin was obtained as a gift sample from MICRO LABS, BANGALORE .The Tablet PERCIN of strength 600 mg were obtained from the market manufactured by LUPIN PHARMA. Muller Hinton Agar and Muller Hinton Broth were obtained from HIMEDIA.

Prulifloxacin Reference Solution

Accurately weighed 5 mg of prulifloxacin was transferred into 50 ml of volumetric flask and dissolved in methanol to obtain a final concentration of 100 µg/ml. Aliquots of this solution were further diluted with buffer no. 1 pH 6 to obtain final concentrations of 20, 30, 40, 50 60 µg/ml.

Preparation of sample solution

Twenty tablets were weighed, crushed and their contents are mixed thoroughly. An accurately weighed portion of powder equivalent to the 10 mg of Prulifloxacin was weighed into a 100 ml volumetric flask containing about 50 ml of Methanol. It was shaken thoroughly for about 5-10 min, and final volume was made with the same. Further filtered thoroughly with Whatman filter paper no. 41 to remove any insoluble matter. Aliquots of this solution were further diluted with buffer no. 1 pH 6 to obtain final concentration.

Microorganism Inoculum

The strain of *Salmonella typhimurium* ATCC 23564 was selected as test organism because of its susceptibility to prulifloxacin and capacity to form sharply defined zone of inhibition, allowing precision in the measurements.

The cultures of *Salmonella typhimurium* ATCC 23564 were cultivated and maintained on Nutrient Agar medium. The organism was grown in a Muller Hinton broth in a sterile test tube, and incubated for 24 hrs at 35 ± 2 °C prior to use.

Agar Diffusion Bioassay

To the sterile 85mm x 10 mm Petri plates 30 ml of Muller Hinton Agar was poured and allowed to solidify. After solidification of this layer, 0.1 ml of *Salmonella typhimurium* ATCC 23564 culture suspension was poured over the layer and was uniformly spread over the entire area. Using sterile cork borer, wells were bored at the centre of the Petri plates. As shown in figure no. 2. 200 µl of drug solution was then filled into the well. Inoculated plates were kept in the refrigerator at 2-8 ^{O} C for 10-15 min for the diffusion of the test solution. The plates were then incubated at 37° C for 24 hrs. At the end of incubation period, inhibition zones formed around the well were measured with transparent scale in millimetre.

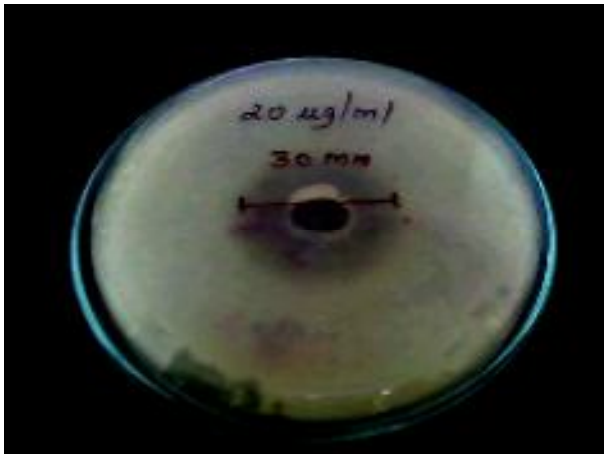

Fig. 2. Microbial assay of Prulifloxacin by agar diffusion method.

Method Validation[12]

The microbiological method was validated as per ICH guidelines Q2 (R1) evaluating for linearity, precision (Method precision, Repeatability) and accuracy.

Linearity

Five doses were used in order to determine the linearity. Calibration plot of zone diameter versus log of concentration was plotted. The regression equation was calculated by least square method.

Precision

Precision was determined by repeatability and method precision and was expressed in terms of relative standard deviation (RSD). Repeatability was carried out by determining sample on the same day were as method precision was done interday on 3 different days for evaluation of the sample considering the same experimental parameters.

Accuracy

Accuracy studies were done as percent recovery; it was performed by adding constant amount of the standard drug to the sample at levels of 80%, 100% and 120% of the test concentration.

Stress degradation studies

Acid degradation

To 2.5 mg of prulifloxacin drug sample 5ml of 0.1 N HCL was added in 25ml volumetric flask and the volumetric flask was kept at normal condition for 3 hour. After 3 hour time interval, solution was neutralized with 0.1 N NaOH and diluted with methanol to attain concentration of 100 µg/ml. Aliquot of this solution was further diluted with buffer no. 1 pH 6 to obtain final concentration.

Alkaline degradation

To 2.5 mg of prulifloxacin drug sample 5ml of 0.1 N NaOH was added in 25ml volumetric flask and the volumetric flask was kept at normal condition for 3 hour. After 3hour time interval, solution was neutralized with 0.1 N HCL and diluted with methanol to attain concentration of 100 µg/ml. Aliquot of this solution was further diluted with buffer no. 1 pH 6 to obtain final concentration.

Thermal degradation

Prulifloxacin drug sample was exposed to temperature of 80 °C for 6 hrs in an oven. After 6 hrs 2.5 mg of drug was diluted to 25 ml with methanol to attain concentration of 100 µg/ml.

Aliquot of this solution was further diluted with buffer no. 1 pH 6 to obtain final concentration.

Photo degradation at 254 nm

Prulifloxacin drug sample was exposed to UV light of 254 nm for 6 hrs in a UV chamber. After 6 hrs 2.5 mg of drug was diluted to 25 ml with methanol to attain concentration of 100 µg/ml. Aliquot of this solution was further diluted with buffer no. 1 pH 6 to obtain final concentration.

RESULTS AND DISCUSSION

The experimental conditions were tested and adjusted to accurately determine the performance of the assay. The strain of *Salmonella typhimurium* ATCC 23564 was found to be an appropriate test microorganism because of its sensitivity to prulifloxacin and its capacity to form sharply defined inhibition growth zones as compared to *Escherichia coli* ATCC 35218 and *Bacillus Subtilis* ATCC 6633.

The bioassay for the drug was performed in triplicate and the mean value is presented. The results of zone of inhibition diameter of prulifloxacin reference substance are tabulated in Table no. 1.

Table no. 1: Results of zone diameter of prulifloxacin reference sample.

Concentration (µg/ml)	log of concentrations (µg/mL)	Zone of inhibition* (mm)
20	1.30	30
30	1.47	33
40	1.60	35
50	1.69	37
60	1.77	38

**average of three determinations*

The calibration curve for prulifloxacin was constructed by plotting zone diameter (mm) versus log of concentrations (µg/mL) which showed good linearity in the range of 20-60 µg/mL as indicated in figure no. 3.

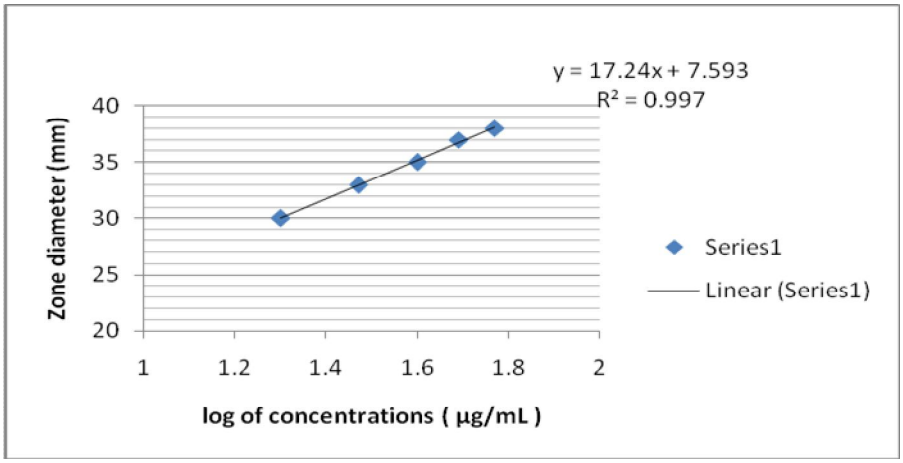

Fig. 3. Calibration curve of prulifloxacin

Linearity and Range

A good linear relation was found between prulifloxacin concentration and zone of inhibition diameter. The regression analysis data is presented in Table no. 2.

Table no. 2: Overview of linearity data

Parameter	Regression analysis data
Regression coefficient	0.997
Intercept	7.593
Slope	17.24
Regression equation	y = 17.24x + 7.593
Concentration range	20-60 µg/ml
Number of points	5

Precision

Method precision

Precision was calculated for Interday. The data obtained shows that method is sufficiently precise. Precision is calculated as % Relative Standard Deviation. Data obtain from precision experiments are given in Table no 3.

Table no. 3: Result of Method precision.

Conc. Of solution (µg/ml)	Zone diameter			Average	% RSD (%)	Conc. from graph (µg/ml)
	Day 1	Day 2	Day 3			
20	30	30	30	30	0	19.93
30	33	33	33	33	0	29.76
50	37	37	37	37	0	50.78

Repeatability

The repeatability of the proposed methods was ascertained by actual determination of six replicates of fixed concentration of the drug within range and finding out the zone diameter by the proposed method. The results are given in Table no 4.

Table no. 4: Result of Repeatability.

Conc (µg/ml)	Zone diameter (mm)	Conc from graph (µg/ml)
20	30	19.93
	30	
	30	
	30	
	30	
	30	
	Mean: 30	
	SD: 0	
	RSD:0	

Accuracy

Accuracy studies were done as percent recovery; it was performed by adding constant amount of the standard drug to the sample at levels of 80%, 100% and 120% of the test concentration. Mean recovery was found to be 95.37% with RSD of 1.74%. The results are tabulated in Table no. 4.

Table no. 4: Results of accuracy by Recovery studies.

Conc. Of sample added (µg/ml)	Level of addition (%)	Conc. Of standard added (µg/ml)	Total Conc. (µg/ml)	Zone diameter *(mm)	Conc. Obtained from graph. (µg/ml)	% Recovery
20	80	16	36	34	34.01	94.47
	100	20	40	35	38.88	97.2
	120	24	44	35.5	41.56	94.45
					Mean	95.37
					SD	1.665
					%RSD	1.74

**Average of three determination*

Stress studies

Prulifloxacin reference sample showed degradation when subjected to thermal stress, alkaline condition, acidic condition and photo degradation. In case of thermal degradation 65.12% of prulifloxacin was degraded, photo degradation caused 84.34 % of degradation, acid

degradation and alkaline degradation resulted in 88.31% and 65.12% degradation respectively. Time duration for carrying out the stress studies were depending upon the prior monitoring of the stress degraded sample at regular intervals of time. Results of stress studies are summarised in Table no. 5.

Table no. 5: Results of stress studies

Stress condition	Time duration (hrs)	Percent degradation (%)
Thermal stress	6	65.12
Photo degradation	6	84.34
Acid degradation	3	88.31
Alkaline degradation	3	65.12

CONCLUSION

This method is successfully validated and can be adopted to show stability of compounding pharmaceutical dosage form containing prulifloxacin. The results indicated that the microbiological cup plate assay demonstrated good linearity, precision and accuracy at concentration ranging from 20 to 60 µg/mL, the method uses simple reagents, less expensive, with minimum sample preparation procedures, and no toxic residues, encouraging its application in routine analysis.

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