METHOD DEVELOPMENT AND VALIDATION FOR QUANTIFICATION OF OZENOXACIN IN PHARMACEUTICAL PRODUCTS BY MICROBIOLOGICAL ASSAY

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ABSTRACT

Ozenoxacin is a novel non-fluorinated topical antibiotic belonging to the quinolone family. HPLC techniques are normally used for the quantification of the efficacy of ozenoxacin, however, the HPLC technique is unable to infer bioactivity against microorganisms. The goal of this article was to augment and authenticate an accurate, sensitive, and yet simple, commercial two-stage cylinder plate (2+2) assay for determining the efficacy and bioactivity of ozenoxacin in pharmaceutical products, which has not been published in any of the scientific journals. This study is based on the development of a bioassay technique for estimating ozenoxacin in pharmaceutical products. In this investigation, the microbiological assay was performed with 08 bacterial strains and 02 fungal strains, from which *Salmonella enterica serotype Abony* MTCC-3858 was selected as the most suitable organism against ozenoxacin based on sharp and clear zone of inhibition. The advanced mathematical approach to linearity, precision and accuracy has been successfully verified and validated. The mean potency recovery value was estimated to be 100.83 % for ozenoxacin cream. In this study, validated methods were used to assess the linearity (r^2=0.9859); the intermediate precision RSD between days was 1.01 %; The mean precision RSD between analysts was 1.04 % and accuracy was 100.67 %, RSD = 0.65 %. Bioassay was optimized by investigating several factors such as robustness by buffer diluent used for standard and sample preparation (phosphate buffer pH 8.0; RSD=0.37 %), inoculum concentration (0.3 %; RSD=0.44 %) and incubation temperature (30 °C; RSD=0.80 %). The result shows that the efficacy of ozenoxacin in pharmaceutical products can be estimated by bioassay technique for quality control purpose.

Keywords: Bioactivity, HPLC technique, Microbiological assay, Ozenoxacin, Potency

INTRODUCTION

Ozenoxacin is chemically [1-cyclopropyl-8-methyl-7-(5-methyl-6-methylaminopyridin-3-yl)-4-oxo-1,4–dihydroquinoline-3-carboxylic acid] (Fig. 1), having a molecular weight 363.41 and the molecular formula of $C_{21}H_{21}N_3O_3$[1].

Ozenoxacin is a non-fluorinated antimicrobial drug of the quinolone family, developed as a 1.0 % cream for the treatment of dermatological disease called impetigo with a strong bactericidal effect against gram-positive bacteria[2]. Ozenoxacin shows bactericidal towards gram-positive bacteria like *Staphylococcus aureus* and *Streptococcus pyogenes* and also exhibits broad spectrum antimicrobial activity against multi-drug resistant strains of *S. aureus* showing resistance towards methicillin, ciprofloxacin and mupirocin antibiotics[3]. The comparative data of some

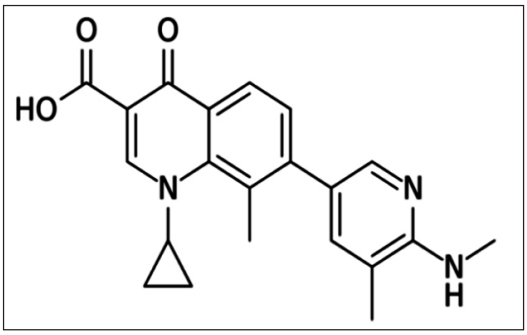

Fig. 1: Chemical structure of ozenoxacin

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in vitro studies proved that ozenoxacin is an effective antimicrobial against *Staphylococci* and *Streptococci*, which are highly contagious skin infections that cause impetigo[3-4]. Ozenoxacin works by blocking the enzymes DNA gyrase and topoisomerase IV, responsible for bacterial DNA synthesis, and reduces the chance of developing antimicrobial resistivity[5]. The efficacy of ozenoxacin has been demonstrated because it hinders the activity of specific ionic pumps located on the surface of *S. aureus*, resulting in the greater aggregation of in gram-positive bacterial cells and the absence of a fluorinated quinolone, which offers a better safety profile like loss of quinolone-induced chondrotoxicity[1,6]. Several physico-chemical techniques, e.g., spectrophotometry and high pressure liquid chromatography (HPLC) were developed for the estimation of ozenoxacin efficacy. Although physico-chemical techniques are fast, accurate and precise, they have some disadvantages, such as interferences with excipients, and others may be difficult to analyze regularly because they are expensive methods that require sophisticated instrumentation and expensive chemicals that are not easily available in various analytical laboratories[7-8]. Quantitative estimation of efficacy is determined through chemical methods such as HPLC, but reduction in antimicrobial activity may not be adequately demonstrated by HPLC. The resistance and susceptibility profile of any antibiotics can be evaluated via utilizing a bioassay method and for calculating the antimicrobial activity of antibiotics[9-12]. In addition, the bioassay can monitor subtle adjustments that can no longer be tested with traditional chemical methods.

The effectiveness as well as bioactivity of the antibiotic can be analyzed by a microbiological assay. For the antibiotic testing cylinder plate method was commonly used, the area of inhibitory zone against different antibiotic dilutions is being tested. Theoretically, in this study, the ratio of the area of inhibitory zone against the different dilutions of antibiotic in a solution is directly proportional and was included to determine the minimum inhibitory concentration (MIC) of the antibiotic in a perforated plate. Another technique called agar diffusion method on solid media is also used for antibiotic susceptibility and resistance calculations and to generate more differentiated responses from calibration zones of microorganisms during MIC evaluation[13]. Accurate assessment of bacterial antibiotic susceptibility is critical to effective bacterial infection management and comparative antimicrobial drug discovery. In order to analyze reproducibility and accuracy of the data from the cylinder plate method, sophisticated statistical methods have also been applied[14]. Bioassay method is a qualitative or quantitative method that helps to determine the exact amount and purity of target drugs or biochemical compounds without involving any residual hindrance of inactive compounds as compared to other analytical methods such as HPLC[9,12,15]. This article presents the study to extend and authenticate a simple and precise agar diffusion microbiological assay technique to estimate the potency of ozenoxacin in cream as an alternative to the HPLC approach.

MATERIALS AND METHODS

Chemical and solvents

Analytical grade chemicals and solvents were used for this study. Reference and test solutions were prepared in dimethyl sulfoxide (DMSO) and distilled water. A working standard of ozenoxacin, which was certified to be 99.54 % pure, was used as a reference substance. A pharmaceutical form, ozenoxacin cream containing 1.0 % (w/w) ozenoxacin was utilized in this experiment.

Equipments

All instruments have been verified for performance through calibrations, these are BOD incubator, laminar air flow, antibiotic zone reader etc. used for bioassay studies. Class B sterilized glassware such as pipettes, volumetric flasks, petri dishes and sterile borer were used in the test. Microbiological culture media have been sterilized through autoclaving for 15 min at 121 °C temperature and 15 psi pressure. For the experiment, glycerol stocks from microbial cultures stored in the freezer were used for microbiological testing. All microbiological assay petri dishes were placed inside BOD for incubation (at 37 °C). Antibiotic zone reader was used to measure the area of the circular zones of inhibition.

Microbial strains

Bacterial and fungal strains were purchased from national culture collection centres such as the Microbial Type Culture Collection (MTCC) and National Collection of Industrial Microorganisms (NCIM), which was equivalent to international culture collection centres as National Collection of Type Culture (NCTC) and American Type Culture Collection (ATCC). These microbial reference cultures are *Escherichia coli* (MTCC 1687 eq. to ATCC-8739), *S. enterica serotype Abony* (MTCC 3858 eq. to NCTC-6017), *Bordetella bronchiseptica* (NCIM 5389 eq. to ATCC-4617), *Pseudomonas aeruginosa* (MTCC 1688 eq. to ATCC-9027); Gram-positive bacteria: *Bacillus subtilis* (MTCC 441 eq. to ATCC-6633), *S. aureus* (MTCC 737 eq. to ATCC-6538P), *S. epidermidis* (MTCC 3615 eq. to ATCC-12228), *Kocuria rhizophila* (MTCC 1541 eq. to ATCC-9341) and Yeast-moulds: *Candida albicans* (MTCC 227 eq. to ATCC-10231) and *Aspergillus brasilliensis*

(MTCC 1344 eq. to ATCC-16404) and were used in bioassay.

Preparation of microbiological medium

The specific media with enrichment properties were used to induce the greater proliferation of the tested organism utilized for testing microbial assay. Culture media was obtained from Hi-Media Ltd., Mumbai. Base layer and seed layer were prepared by Antibiotic Assay Medium No. B. The composition of the media contained 6.0 g L^{-1} peptone, 3.0 g L^{-1} yeast extract, 1.5 g L^{-1} HM peptone B and 15.0 g L^{-1} agar powder; final pH was adjusted at 6.55±0.05. Fresh slants of tryptone soya media (TSA) and Sabouraud dextrose agar (SDA) have been utilized for bacterial and fungal growth respectively. Distilled water used for media preparation was sterilized by autoclaving for 15 min at 121 °C temperature and 15 psi pressure. The final pH was adjusted according to the instructions on the media container.

Preparation of reference standard

The reference standard of ozenoxacin was weighed in an accurate amount of 10.0 mg and dissolved in 100 mL of dimethyl sulphoxide to obtain 100 µg mL^{-1} of ozenoxacin. During the experiment, different dilutions were freshly prepared from reference standard diluted in distilled water viz. 10 µg mL^{-1}, 5.0 µg mL^{-1}, 4.0 µg mL^{-1}, 2.0 µg mL^{-1}, 1.0 µg mL^{-1}, 0.5 µg mL^{-1} and 0.1 µg mL^{-1}. The concentrations of reference solution 4.0 µg mL^{-1} and 1.0 µg mL^{-1}, both with dilution ratio of 4:1 was chosen as reference standard high (R_1) and reference standard low (R_2), respectively.

Ozenoxacin test solution preparation

A quantity of 1.0 g of cream equivalent to 10 mg ozenoxacin is accurately weighed and dispensed into a volumetric flask of capacity 100 mL. Dimethyl sulphoxide (DMSO) of 50 mL quantity has been added to proper dissolution of the sample and then the volume was madeup for 100 mL capacity to obtain 100 µg mL^{-1} stock solution with dimethyl sulphoxide. Stock solution of the test was diluted in the ratio of 4:1 dilution in the distilled water to get the test higher concentrations (T_1= 4.0 µg mL^{-1}) and the lower test concentration (T_2= 1.0 µg mL^{-1}) used for the test.

Standardization of microbial inoculum preparation

Pure microbial cultures were revived from glycerol stocks and plated over the freshly prepared slants of tryptone soya agar (TSA) and Sabouraud dextrose agar (SDA) slants to induce the bacterial and fungal growth. Tryptone soya agar slants were incubated for 24-48 h at 37˚C for the incubation of bacteria and Sabouraud dextrose agar slants were incubated at 20-25 °C for 72-120 h for fungal growth. After incubation, they were washed with 3 mL of sterile saline (0.9 %) to harvest the growth of the organism from the surface of slant and was diluted the appropriate amount of harvest suspension to determine the target value measured using UV spectrophotometer, which gave a transmission of about 25 % at 530 nm. This diluted inoculum was stored refrigerated and used for further microbiological assay experiments.

Agar diffusion bio assay method

The two-level factorial microbiological assay of cup plate method was performed in quadruplicate. The cup plate assay is based on the diffusion method where antibiotics diffuses from a vertical cup or cavities via layer of solid agar in a petri-plate. The growth of the microorganism spread in the solid agar plate is restricted nearby circular well area around the cup/cavities containing antibiotic solution[9]. 21 mL of test medium was inoculated in a Petri dish (100 mm diameter) and allowed to set to a smooth base layer of uniform depth. After the solidification, the determined target value of the suspension of microorganisms was added to the seed layer agar medium to prepare bilayer plates of the assay by pouring 4 mL to spread the inoculum over a solidified base layer surface and let it solidify. These plates were left for 30 min to solidify. Four circular holes with a diameter of 8 mm were bored into the solidified agar plate using a sterile borer. These wells have been labeled as a reference standard and test solution of low and high concentration. Using a micropipette, these marked wells were filled with 100 µL of low and high concentration reference standard solutions. Equally low and high concentrations of test solutions corresponding to the reference standard solution were filled into the wells. Agar petri plates filled with solutions were left at room temperature for 1-4 h to reduce the effects of the time difference between each tested plate dilutions. Then agar plates were subjected to incubation for 18-24 h at 37 ˚C. The zone of inhibition was accurately measured upon completion of incubation, with the help of antibiotic zone reader with the diameter (mm) and the results were calculated for further interpretations (Fig. 2). The percentage antibiotic-potency of ozenoxacin cream relative to the reference standard was calculated via using Indian Pharmacopoeia's model equation.

$$\% \text{ Potency} = \text{Antilog } (2.0 \pm a \log I)$$

$$\text{in which, } a = \frac{(T_1+T_2) - (R_1+R_2)}{(T_1-T_2) + (R_1-R_2)}$$

T_1 and T_2 are the sum of the zone diameters with high and low level Test solutions, R_1 and R_2 are the sum of the zone diameters with higher level and lower level of reference standard solutions, and I = dilution ratio.

Fig. 2: Zone of inhibition by two level factorial assay against

S. enterica serotype Abony MTCC-3858

(Reference Standard solutions R_1= 4.0 µg mL^{-1}, R_2= 1.0 µg mL^{-1},

Test solutions T_1= 4.0 µg mL^{-1}, T_2= 1.0 µg mL^{-1})

RESULTS

Selection of most suitable microorganism

The selection of the most suitable microorganism was based on sharp and clear margins and a broader zone of inhibition under antibiotic influence. A microbiological test for ozenoxacin was carried out on 08 bacterial strains and 02 fungal strains with regard to their reaction and sensitivity.

The result show that the growth of strains *B. subtilis* (MTCC-441), *C. albicans* (MTCC-227) and *A. brasilliensis* (MTCC-1344) was not inhibited by ozenoxacin and showed no inhibitory effect. *S. aureus* (MTCC-737), *K. rhizophila* (MTCC-1541) and *B. bronchiseptica* (NCIM-5389) showed an intermediate zone of inhibition. However, *E. coli* (MTCC-1687) and *S. enterica serotype Abony* (MTCC-3858) were susceptible, while *S. epidermidis* (MTCC-3615) and *P. aeruginosa* (MTCC-1688) showed a large and light zone (Table I). *S. enterica serotype Abony* (MTCC-3858) was observed to have a highest and significant zone of inhibition against ozenoxacin. Therefore, *S. enterica serotype Abony* (MTCC-3858) was selected as the most suitable organism and used for further bioassay studies.

Table I: Selection of most suitable microbes for microbiological assay of ozenoxacin

Name of microbe	Area of zone of inhibition (mm) at 4 µg mL^{-1}	Interpretation
K. rhizophila (MTCC-1541)	13.5	Intermediate zone
B. bronchiseptica (NCIM-5389)	11.2	Intermediate zone
S. aureus (MTCC-737)	15.4	Intermediate zone
E. coli (MTCC-1687)	19.1	Sharp zone
S. enterica serotype Abony (MTCC-3858)	19.2	Very sharp and clear zone
S. epidermidis (MTCC-3615)	20.3	Large and light zone
P. aeruginosa (MTCC-1688)	24.1	Large and light zone
B. subtilis (MTCC-441)	-	No inhibition zone
C. albicans (MTCC-227)	-	No inhibition zone
A. brasilliensis (MTCC-1344)	-	No inhibition zone

Determination of optimal inoculum concentration

In the present study, a test was carried out on how to choose the concentration of the inoculum. An optimal inoculum concentration was selected based on sharp zones. Low inoculum concentration implies lower colonies and an unusually large zone diameter, while very high concentration of bacterial culture inocula indicates an overlapping of colonies and poorly developed zone diameter. The optimal concentration of culture inocula must be within recommended two limits, prior optimized at 25 % transmittance; six different concentrations of inoculants, with 0.05 %, 0.1 %, 0.2 %, 0.4 %, 0.5 % and 1.0 % dilutions were used to test their action on area of inhibition zones (Table II). The optimal inoculum concentration of *S. enterica serotype Abony* (MTCC-3858) was decided to be 0.2 % for performing the antibiotic assay.

Table II: Interpretation of area of zone of inhibition under various concentrations of inoculants

Inoculum concentration (%)	Antibiotic Conc. (µg mL^{-1})	Zone of Inhibition area (mm)	Interpretation
0.05	4	-	Very light and large zone
0.1	4	22.2	Light and large zone
0.2	4	21.5	Very clear and sharp edge zone
0.4	4	17.1	Zone without sharp edge
0.5	4	18.5	Overlapped zone with cloudy growth
1.0	4	17.4	Overlapped zone with cloudy growth

Evaluation of the optimal concentration of antibiotics

The minimum inhibitory concentration of the antibiotic is an essential parameter that prevents the growth of microbes. The reference solution concentration was estimated based on a clear, sharp edge and a measurable zone size. Different concentrations, i.e. 10 µg mL^{-1}, 5.0 µg mL^{-1}, 4.0 µg mL^{-1}, 2.0 µg mL^{-1}, 1.0 µg mL^{-1}, 0.5 µg mL^{-1}, 0.1 µg mL^{-1} dilutions from the 100 µg mL^{-1} reference stock of ozenoxacin were prepared. A concentration of 4 µg mL^{-1} of reference solution has been found to gives a clear, sharp edge and a measurable area of zone of inhibition. The outcome of various concentrations of the ozenoxacin reference standard on zone of inhibition is shown in Table III.

Table III: Influence of ozenoxacin reference standard on area of inhibition zone

Reference standard concentration (µg mL^{-1})	Area of inhibition zones (mm)
10	24.8
5	22.6
4	21.5
2	20.1
1	17.9
0.5	14.2
0.1	Non measurable

Calculation of % potency

All experiments were performed in triplicates. The antibiotic zone reader was used to measure the area of the circular inhibition zones of the standard and test solutions at higher and lower concentrations. The mean percent efficacy was estimated to be 100.83 % for ozenoxacin cream using a standard equation.

Method validation

All microbiological assay parameters have been validated precisely to assess the outcome of the microbiological assay method. According to International Conference on Harmonization[16-17], Microbiological test has been evaluated through the statistical parameters (linearity, range, specificity, accuracy and robustness).

Linearity - Linearity of the bioassay was measured at various dilutions, i.e. 1.0, 2.0, 4.0, 5.0 and 10 µg mL^{-1} of ozenoxacin reference standard solution. Least squares regression analysis model has been used for the result interpretation against a log concentration (log 10) of ozenoxacin in (µg mL^{-1}) plotting calibration curve against area of inhibition zone (mm). In the regression analysis, the determined linear equation was Y = 1.63 x + 16.49 and the regression coefficient was (r^2 = 0.9859) (Fig. 3).

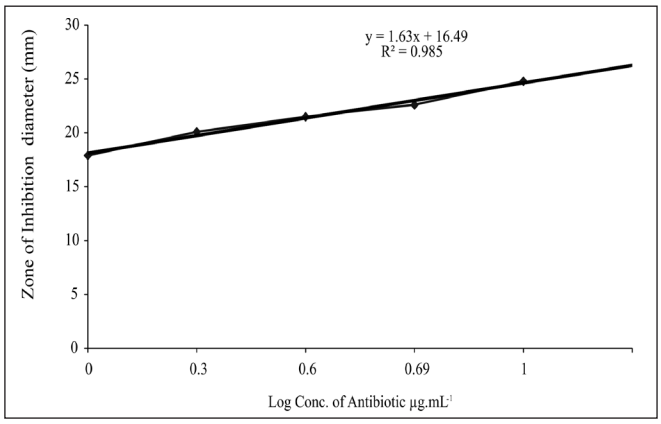

Fig. 3: Standard curve for ozenoxacin concentration determined from antibiotic assay

Range - The range was calculated from selective concentrations of the calibrated reference solution used to plot the standard curve verified through linearity, specificity and accuracy of assay.

Specificity - Relative standard deviations (RSD) were used for various precision parameters, i.e., repeatability and intermediate precision were calculated. The repeatability of ozenoxacin cream was determined in six replicates on very day by specific analyst (Table IV).

The intermediate precision was calculated by repeating the test on two consecutive days (between days) and between different analysts (between analysts) (Table V).

Table IV: Repeatability for ozenoxacin cream by bioassay

Sample	Stated amount (%w/w)	Experimental amount (%w/w)	% Potency	Mean % potency	RSD (%)
Ozenoxacin cream	1	1.02	102.0	100.83	1.15
		1.004	100.37		
		0.993	99.30		
		0.998	99.83		
		1.015	101.46		
		1.020	102.02		

Table V: Intermediate precision for ozenoxacin cream by bioassay

Precision	Experimental % potency	Mean % potency	RSD (%)
Inter-day Precision			
Day 1	98.30	99.69	1.01
	100.63		
Day 2	99.68		
	100.14		
Inter-analyst Precision			
Analyst 1	97.75	99.22	1.04
	99.83		
Analyst 2	100.02		
	99.27		

Accuracy - For the microbiological assay method, the accuracy was evaluated between 80 %, 100 % and 120 % of minimal assay dilutions. The calculated average percentage accuracy was (100.67 %; RSD 0.65 %), indicating that the approach can accurately determine ozenoxacin concentrations in the 80, 100, and 120 % range (Table VI).

Robustness - Robustness was determined after examining the same material under different settings. Different parameters were considered for its estimation like concentration of different microbial inoculants; buffers used for standard preparation were all taken into account. To investigate resilience, a number of factors were changed from the usual assay settings, including the buffer diluent used for standard and sample preparation (phosphate buffer pH 8.0), inoculum concentration (0.3 %) and incubation temperature (30 °C). As shown in Table VII, no significant variations in potencies were found when the experimental settings were changed to the required specifications.

Table VI: Accuracy for ozenoxacin cream by bioassay

Hypothetical % potency	Experimental % potency	Mean % potency	Accuracy (%)	RSD (%)
80	81.36	81.09	100.67	0.65
	80.42			
	81.49			
100	100.28	100.07		
	100.35			
	99.58			
120	121.19	120.71		
	120.54			
	120.39			

Table VII: Factors affecting *in vitro* culture parameters assessed robustness

Factor	Parameter	Mean % potency	RSD (%)
Solvent	Phosphate buffer pH 8.0	100.59	0.37
		99.85	
		100.15	
Inoculum concentration	0.3 %	100.53	0.44
		99.66	
		100.12	
Incubation temperature	30 °C	99.28	0.80
		100.33	
		100.84	

DISCUSSION

The selection of an appropriate analytical approach is critical for effective drug control and is influenced by a variety of parameters such as the source of the drug, its composition, amount of prepared reagents and equipment performance comprising data with research article survey. Several studies have confirmed the application of various analytical instruments like HPLC and various biochemical techniques to demonstrate the potency of ozenoxacin. In the various literature studies, there is currently no

established microbiological assay technique for evaluating ozenoxacin activity.

Despite the fact that HPLC is commonly used to measure the effectiveness of antibiotics, it cannot determine the bioactivity of antibiotics against microorganisms. On the other hand, microbiological assays can show both the bioactivity and the potency of an antibiotic. The outcome of the antibiotic assay can be utilized for the predictive dose assessment of antibiotics for resistant bacteria. Consequently, this study helps us to cross verify the bioassay efficacy in a most convenient and simple approach to estimate ozenoxacin in pharmaceutical product data used in drug quality control[18]. Antibiotic assays are able to assess the accurate dose of specific antibiotics against resistant bacteria with a least risk of sample handling and biochemical errors. However, the potency and bioactivity of antibiotics in medicines and biochemical products can be improved with the precise validation of several parameters. Thus, the inactive elements associated with them do not intervene, preserving the accuracy of the analytical approach[19]. Therefore, in general, the bioassay remains the standard for clarifying doubts regarding a possible loss of activity[10]. Some articles present the bioactivity of ozenoxacin against bacterial strains but do not describe the technical details of estimating ozenoxacin activity in pharmaceutical products.

In this study, a microbiological test for ozenoxacin was performed on 08 bacterial strains and 02 fungal strains towards antibiotic susceptibility or resistance. Microbial strains used in this study show a positive reaction, i.e. exhibited sensitivity to ozenoxacin, but due to the ability to form sharp-edged zones of inhibition, *S. enterica serotype Abony* MTCC-3858 was selected as the most appropriate test organism. Based on a clear and sharp-edged zone of inhibition, the inoculum concentration for quantification of an antibiotic should be validated throughout the microbial bioassay[14]. Bioassays have been tested to assess the inoculants dilution while other parameters are kept constant, as it is well documented that the concentration of the inoculum affects the resulting zone size[14,20]. Petri dishes containing media with a higher dose of the test micro-organism show turbid characteristics in the growth media and show no antimicrobial effect of the antibiotic, while a low culture inoculant of the tested micro-organism produces a hazy to light or unmeasurable zone. Therefore, it is necessary to optimize the inoculum concentration for microbiological assays. In this study, the different dilutions of 0.05 %, 0.1 %, 0.2 %, 0.4 %, 0.5 % and 1.0 % of tested micro-organism, i.e., *S. enterica serotype Abony* MTCC-3858 were tested and optimized to an inoculum concentration of 0.2 % microbes set bioassay. In the proposed bioassay, a series of specific dilutions of ozenoxacin as a reference standard was taken to measure the zone of inhibition. Different concentrations i.e. 0.1, 0.5, 1.0, 2.0, 4.0, 5.0 and 10 µg mL^{-1} of reference standard of ozenoxacin were tested against selected test microorganisms. The reason for the chosen concentration was the susceptibility of the microbes to minimum concentrations of selected antibiotic, and the mean area of zones of inhibition to high concentrations, and showing the linear association between the logarithmic concentration and the average area of inhibition zone, which is restricted by the petri-plate size. Good linearity was observed across the indicated concentrations of the ozenoxacin reference standard by measuring the standard curve under the log concentration (log10) of the antibiotic dilutions (µg mL^{-1}) versus the average area of zone of inhibition (diameter in mm). Accuracy of plotted area will be expressed as relative standard deviation (RSD). Reproducibility of the test results was determined by analyzing ozenoxacin cream multiple times (same day/time). The average value of ozenoxacin concentration in the cream was reported to be 100.83 % (RSD 1.15 %). In the day-wise precision assay, the average value of ozenoxacin level was 99.69 % with an RSD of 1.01 % and between analysts the mean level was 99.22 % (RSD of 1.04 %). Accurate measures for the antibiotic assay have been estimated between 80 % to 120 % of the minimum inhibitory concentration and with an average precision of 100.67% (RSD 0.65%), guaranteeing the reliability of the bioassay. The usefulness of the antibiotic assay method for evaluating the quality of drugs and biochemicals products from pharmaceutical origin was proven by this study.

CONCLUSION

To determine the quality of pharmaceutical products, the use of validated and authenticated analysis methods is absolutely necessary. HPLC methods were developed and used to estimate the potency of ozenoxacin in the various references. However, there is no authenticated microbiological method for estimating the potency of ozenoxacin that has been published in any literature. Antibiotic bioassay acts as a verified procedure for the clarification of the antibiotics inactivity. Main advantage of these microbiological assays is to quantify the titre of an antibiotic over HPLC methods, despite the fact that both the techniques are efficient and shows equivalency in their outcomes. It appears to be a better technique due to the assessment of both potency and bioactivity through microbiological testing. The bioactivity and assessment of the effectiveness of an antibiotic using a microbiological test procedure is simple, precise, sensitive

and also economical to operate. The planned experiment was optimized to check and estimate the effectiveness of the ozenoxacin antibiotic assay in a bioassay under different parameters and culture conditions. It was found that *S. enterica serotype Abony* MTCC-3858 is the most susceptible organism to ozenoxacin. Different culture parameters like diluent type and its pH, inoculants dilution, reference and test dilutions were examined in test organism *S. enterica serotype Abony* MTCC-3858 against ozenoxacin. Potency of ozenoxacin in a cream sample was estimated by bioassay to be 100.83 %. The results showed that the proposed microbiological test procedure for ozenoxacin is beneficial for estimating the potency of ozenoxacin in pharmaceutical products.

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