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Assessment of the Potency of Ceftiofur Sodium Powder through Validated Microbiological Method

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Abstract

Ceftiofur Sodium is a semi-synthetic beta-lactamase broad spectrum third generation of cephalosporin antibiotic. The development and validation of a two-level agar diffusion (2+2) bioassay to quantify ceftiofur in powder for injection is described in this paper. Bacterial strain *Bacillus subtilis* ATCC 6633 (MTCC 441) was selected and used as the most significant strain against ceftiofur sodium. The mean potency recovery value of ceftiofur sodium in marketed sample XCEFT powder for injection was estimated to be 101.79%. All potency results were statistically analyzed and found to be linear (r^2 = 0.9916) in the range of 1.0-10 μgmL^{-1}, with the intermediate precision RSD between days was 0.81%; intermediate precision RSD between analyst was 0.43% and accuracy 101.08%, RSD = 0.28%. The findings backed up the proposed microbiological technique, which allowed for accurate ceftiofur sodium quantification in pharmaceutical samples. Furthermore, bioassay is a useful, easy and cost-effective method for controlling the quality of ceftiofur sodium in raw material as well as in pharmaceutical preparations.

Keywords: Ceftiofur sodium, *Bacillus subtilis*, Bioassay, Quality Control, Potency.

Introduction

Ceftiofur sodium is a semi synthetic, beta-lactamase broad spectrum third generation cephalosporin group antibiotic, approved for use in veterinary medicine by Food and Drug Administration (FDA). Ceftiofur sodium has been developed as dry powder injection which is reconstituted in sterile water prior to intramuscular administration. Ceftiofur is used to treat respiratory disease in swine, ruminants and horses associated with *Actinobacillus (Haemophilus) Pleuropneumoniae, Pasteurella multocida, Pasteurella himolytica, Salmonella choleraesuis* and *Streptococcus suis*[1-4]. Ceftiofur is resistant to beta lactamase producing organisms and has an antibacterial activity against both Gram-positive and Gram-negative bacteria.[4,5] Escherichia coli strains resistant to ceftiofur have been reported.[6]

Ceftiofur sodium is sodium 7-[(Z)-2-(2-amino-1,3-thiazol-4-yl)-2-methoxyimino)acetamido]-3-(2-furoylthiomethyl)-3-cephem-4-carboxylate (Fig. 1) with the chemical formulae $C_{19}H_{16}N_5NaO_7S_3$ and a molecular weight of 545.5. It is an off-white crystalline powder.[7]

The pharmacopoeias recommend using high performance liquid chromatography (HPLC) with UV detection and a mobile phase composed of water, phosphate buffer, and acetonitrile for official quality control of ceftiofur sodium as a raw

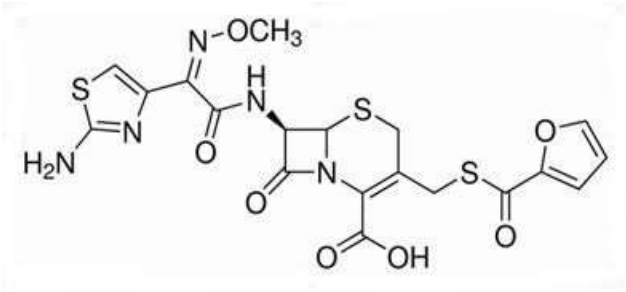

Fig. 1: Chemical Structure of Ceftiofur sodium

ingredient or in pharmaceutical preparations.[7]

There is only one method reported in literature which employs a 3×3 agar diffusion assay using *Micrococcus luteus* (ATCC 10240) as the test organism for their microbiological assay for the quantitative determination of ceftiofur sodium powder.[8] However, two level (2×2) factorial agar diffusion microbiological assay method, which was validated in this present study for quantitative determination of ceftiofur sodium in dry powder injection, is mentioned only in the Indian Pharmacopoeia.[7] Several methods for determining ceftiofur in cow milk and serum using high performance liquid chromatography have been reported in the literature.[9-10]

Microbiological assay for other antibiotics such as streptomycin, spiramycin, tylosin and oxytetracycline used in veterinary practice have been described in Indian Pharmacopoeia.[7] Because ceftiofur sodium is used to treat bacterial infections in animals, alternative methods for quantification in pharmaceutical dosage forms must be developed. To calculate the antimicrobial activity of antibiotics using bioassay method, their inhibitory effect on the growth of the test microorganisms is evaluated.[7,11-13] Moreover, the bioassay can monitor subtle adjustments which can no longer be detected by means of traditional chemical approach. The bioactivity as well as the potency of an antibiotic can be estimated by microbiological assay. The cylinder plate method extensively used in assay of antibiotics, correlates the area of inhibition with the dose of the antibiotic being tested. This study has taken in to account the theoretical relationship between the area of inhibition zone and the concentration of antibiotic in a solution carried out in perforated plate. Practically the area of inhibition zone is directly proportional to the antibiotic concentration.

The reproducibility and precision of data from the cylinder plate method have led to the development of advanced statistical techniques.[14] The agar diffusion method can also be used to create calibration zones that isolate the range of susceptible, resistance, or confer with more sophisticated responses of microorganisms to antibiotic drugs.[15] Microbiological assay strategies provide an appropriate amount of antibiotic potency while posing only a minor risk of interference from degradation products or biologically inactive components.[16]

This article presents a study to develop and validate an easy and precise microbiological assay by agar diffusion technique for quantitation of ceftiofur sodium as a raw material and injectable formulation, as an alternative to the physicochemical methods prescribed in pharmacopoeias.

Material and Methods

Chemicals

A working standard of ceftiofur sodium (purity assigned 98.0%) was used as a reference substance, and a commercially sample XCEFT dry powder for injection containing ceftiofur sodium 250 mg per vial was obtained from the market. Reference and test solution were prepared in sterile distilled water.

Equipment

Calibrated and validated equipment was used for bioassay study. Sterilized glassware of Class B such as pipettes, volumetric flasks, petri plates and sterile borer were used during the test. Microbiological media was sterilized at 121 °C and 15 psi for 15 min in an autoclave. For the microbiological assay, glycerol stocks of microbial cultures stored in deep freezer were used. Petri plates of microbiological assay were incubated at 37 °C in BOD Incubator. Antibiotic zone reader was used for measuring the area of circular inhibition zones.

Microbial strains

Bacterial strains were purchased from national culture collection centre such as Microbial Type Culture Collection (MTCC) and National Collection of Industrial Microorganisms (NCIM) which was equivalent (eq.) to international culture collection centre as American Type Culture Collection (ATCC) and National Collection of Type Culture (NCTC). Total 9 bacterial strains were used in bioassay among them 4 are Gram-negative and 5 are Gram-positive bacteria. The Gram-negative bacteria are *Escherichia coli* (MTCC 1687 eq. to ATCC-8739), *Salmonellae 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) and Gram-positive bacteria are *Bacillus subtilis* (MTCC 441 eq. to ATCC-6633), *Staphylococcus aureus* (MTCC 737 eq. to ATCC-6538P), *Staphylococcus aureus* (MTCC 96 eq. to ATCC-9144), *Staphylococcus epidermidis* (MTCC 3615 eq. to ATCC-12228), *Kocuria rhizophila* (MTCC 1541 eq. to ATCC-9341).

Microbiological media

The purpose of the media is to promote the quick development of the tested organism being used in the bioassay. Media used for bioassay were procured from Mumbai. Base layer and seed layer was prepared using an Antibiotic Assay Medium No. B. The composition of the media contained 6.0 gL^{-1} peptone, 3.0 gL^{-1} yeast extract, 1.5 gL^{-1} HM peptone B and 15.0 gL^{-1} agar powder; final pH was adjusted at 6.55 ± 0.05. For bacterial growth, fresh slants of tryptone soya agar were used. Distilled water was used for media preparation and sterilized in an autoclave at 121°C and 15 psi for 15 min. The final pH was adjusted in accordance with the instructions on the media container.

Solution of reference substance

A sufficient quantity of reference substance of ceftiofur sodium was accurately weighed and dissolved in 25 mL sterile distilled water to obtain 1000 μgmL^{-1}. The stock solution was kept in a refrigerator. On the day of the experiment, different dilutions viz. 10, 5.0, 4.0, 2.0 and 1.0 μgmL^{-1} were prepared in sterile distilled water from the stock solution. The concentrations of reference solution 4.0 μgmL^{-1} and 1.0 μgmL^{-1}, both with a 4:1 dilution ratio were chosen as reference standard high (S_H) and reference standard low (S_L), respectively.

Sample solution

To make a 1000 μgmL^{-1} stock solution of sample, 250 mg of XCEFT powder for injection sample was diluted in 250 mL of sterile distilled water. Aliquots of this stock test solution were diluted in ratio of 4:1 in distilled water to get the sample high concentrations (T_H = 4.0 μgmL^{-1}) and the sample low concentration (T_L= 1.0 μgmL^{-1}), which were used in bioassay.

Standardization of inocula preparation

Glycerol stocks of bacterial cultures were revived and cultured on the slants of Tryptone soya agar to maintain the growth of bacteria. Tryptone soya agar slants were incubated for 24-48 h at 37 °C. After incubation, washed out with 3 mL sterile saline (0.9%) to harvest the growth of organism from the surface of agar slants and diluted appropriate amount of harvest suspension to determine the target value which gave approximately 25% transmission at 530 nm using UV spectrophotometer. This diluted inoculum was stored under refrigeration and used for further experiments of microbiological assay.

Bioassay method using agar diffusion

The cup plate method for two-level factorial microbiological assay was carried out in quadruplicate. The cup plate method relies on antibiotic diffusion through a solidified agar layer in a Petri plate through a vertical cup or cavities. The growth of the specific microorganism inoculated in the agar is prevented in a circular area around the cup or cavities containing the solution of the antibiotic.[7] In a 100 mm × 20 mm Petri dish, pour 21 mL un-inoculated base layer of assay medium and allow it to harden in to a smooth base layer of uniform depth. After solidification, determined target value of suspension of microorganisms was added to seed layer agar medium to prepare double-layer plates of assay by pouring 4 mL to spread the inoculums uniformly over a solidified base layer surface and allow to solidified.[11] These plates were left to solidify for at least 30 min. Four circular holes were bored having a diameter of 8 mm in to the solidified agar plate with the help of sterile borer. These holes were marked as low and high concentration with respect to reference and sample solutions. Through micropipette, these labelled holes were filled with 100 µL reference substance and sample solutions of low and high concentration respectively. To minimize the impact of time differences between the applications of the different solutions, agar petri plates loaded with solutions were left at room temperature for 1-4 h. Then agar petri plates were incubated for 18-24 h at 37 °C in BOD incubator. After completion of the incubation period, diameters (mm) of zone of inhibition were accurately measured through antibiotic zone reader and calculate the results accordingly (Fig. 2). All of the experiments were carried out in a Biosafety Cabinet.

The percentage potency of the XCEFT injection was calculated using the Indian Pharmacopoeia's model equation.

$$\text{Percentage potency} = \text{Antilog}\,(2.0 \pm a \log I)$$

$$\text{In which, } a = \frac{(T_H+T_L) - (S_H+S_L)}{(T_H-T_L) + (S_H-S_L)}$$

T_H and T_L are the sum of the zone diameters with

Fig. 2: Two level agar diffusion assay method using a test microorganism Bacillus subtilis ATCC-6633 (MTCC 441) at concentration of Reference solution SH = 4 μgmL^{-1}, S_L = 1 μgmL^{-1} and Sample solution S_H = 4 μgmL^{-1}, S_L = μgmL^{-1}).

high and low level sample solutions, S_H and S_L are the sum of the zone diameters with high and low level reference standard solutions, and I = dilution ratio.

Results

Selection of most suitable microorganism

Most suitable microorganism was selected on the basis of sharp & clear edges and large measurable zone diameter under antibiotic behaviour. Microbiological assay of ceftiofur sodium was performed on 09 strains of bacteria for their response and susceptibility.

Result shows that microbial strains B. *bronchiseptica* (NCIM-5389), *P. aeruginosa* (MTCC-1688), *S. aureus* (MTCC-737), *S. epidermidis* (MTCC-3615) *S. enterica serotype abony* (MTCC-3858) growth were not inhibited by ceftiofur sodium and do not show any inhibitory effect. *S. aureus* (MTCC-96) and *E. coli* (MTCC-1687) were susceptible against ceftiofur sodium and shows intermediate zone of inhibition. However, *K. rhizophila* (MTCC-1541) showed large and light zone where as *B. subtilis* (MTCC-441) exhibit a highest and considerable inhibition zones against ceftiofur sodium (Table I). Therefore, B. *subtilis* (MTCC-441) was selected as the most appropriate organism and used for further bioassay study.

Determination of optimal inoculum concentration

In the current study, a test was conducted to determine how to choose the inoculum concentration. On the basis of sharp and clear zones, optimal inoculum concentration was selected. Low inoculum concentration implies poor growth and an unusually large zone diameter, whereas excessive inoculum concentration indicates an overlapping growth pattern and a lower zone diameter. Optimal inoculum concentration should lie between these two limits.

Five different inoculums concentrations, i.e., 0.5%, 1.0%, 1.5%, 2.0% and 3.0% were used in this study to test their influence on area of inhibition zones, which was previously optimised at 25% transmittance (Table II). For the microbial bioassay, the optimal inoculum concentration of *B. subtilis* (MTCC-441) was selected as 2.0%.

Table I: Selection of most suitable microbes for microbiological assay of ceftiofur sodium.

Name of microbes	Zones of inhibition (mm) of growth at 4 μgmL^{-1}	Interpretation
Staphylococcus aureus (MTCC-96)	10.1	Intermediate zone
Escherichia coli (MTCC-1687)	13.2	Intermediate zone
Kocuria rhizophila (MTCC-1541)	24.1	Large and light zone
Bacillus subtilis (MTCC-441)	19.1	Sharp and Clear zone
Staphylococcus epidermidis (MTCC-3615)	-	No inhibition zone
Bordetella bronchiseptica (NCIM-5389)	-	No inhibition zone
Pseudomonas aeruginosa (MTCC-1688)	-	No inhibition zone
Salmonellae enterica serotype abony (MTCC-3858)	-	No inhibition zone
Staphylococcus aureus (MTCC-737)	-	No inhibition zone

Table 2: Action of different inoculum concentration on the area of growth inhibition zones.

Inoculum concentration (%)	Antibiotic Conc. (μgmL^{-1})	Area of growth inhibition zones (mm)	Interpretation
0.5	4	22.5	Light and overlapped zone
1.0	4	21.2	Light zone
1.5	4	20.1	Zone without Sharp edge
2.0	4	19.5	Very clear and sharp edge zone
3.0	4	18.1	Sharp Zone

Determination of optimal antibiotic concentration

The concentration of antibiotic is a critical factor that prevents the growth of microbes. On the basis of clear, sharp edge and measurable zone size, the concentration of reference solution was estimated. From the 1000 μgmL^{-1} standard stock solution of ceftiofur, different concentration of 10, 5.0, 4.0, 2.0 and 1.0 μgmL^{-1} were made. It was determined that a concentration of 4 μgmL^{-1} of reference solution gave a clear, sharp edge and measurable zone. Table III shows the effect of various concentrations of the ceftiofur reference substance on zone inhibition.

Estimation of percentage potency calculation

For all experiments, triplicate plates were used. The area of the circular inhibition zones of reference and sample solutions at high and low concentration levels was measured using an antibiotic zone reader. The Pharmacopoeia's standard model equation estimated the mean percentage potency for XCEFT powder for injection as 101.79%.

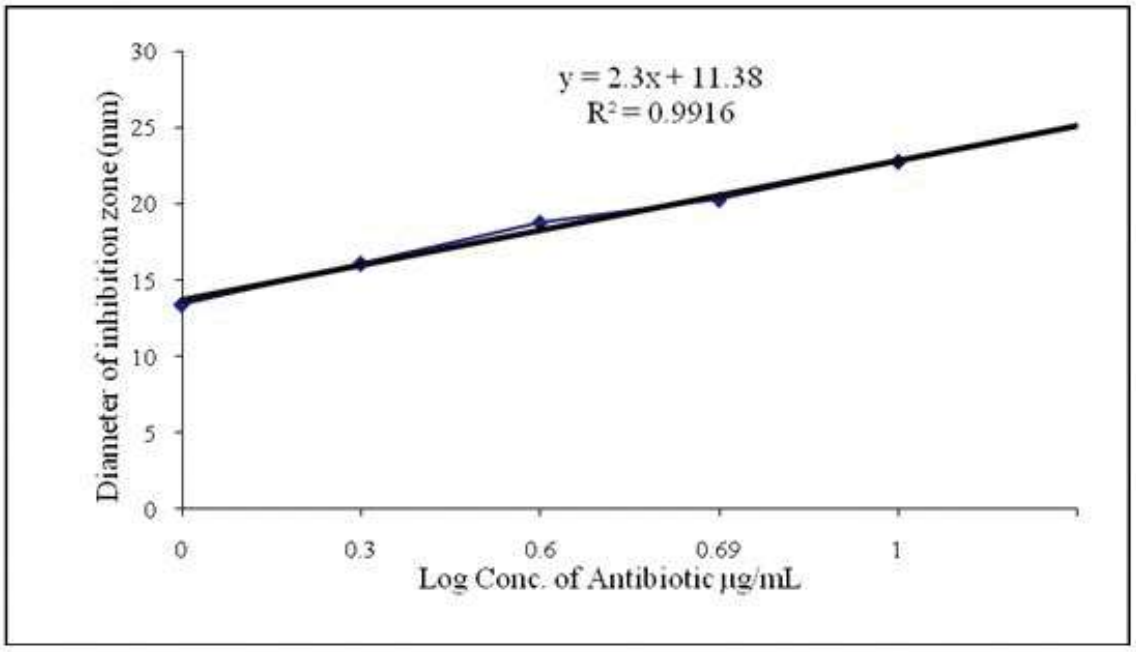

Fig. 3: Bioassay derived calibration curve for ceftiofur sodium reference substance

Table 3: Effect of ceftiofur reference substance concentrations on inhibition zone.

Reference substance concentration (μgmL^{-1})	Area of growth inhibition zones (mm)
10	22.8
5	20.3
4	18.8
2	16.1
1	13.4

Table 4: Repeatability evaluation for XCEFT powder for injection by bioassay.

Stated amount (mg/vial)	Experimental amount (mg/vial)	% potency	Mean % potency	RSD (%)
250 mg	251.15	100.46	101.79	1.07
	254.88	101.95		
	252.15	100.86		
	258.60	103.44		
	255.88	102.35		
	254.13	101.65		

Method validation

Prior to validation, all microbiological assay parameters were optimised in order to properly evaluate the performance of the proposed microbiological assay method. The microbiological assay method was validated using the International Conference on Harmonization's criteria for linearity, precision, accuracy, and robustness.[17,18]

Linearity

The linearity of the bioassay was measured using concentrations of 1.0, 2.0, 4.0, 5.0 and 10 µgmL-1 of ceftiofur sodium reference substance solution. The data were subjected to least squares regression analysis after plotting a calibration curve for log10 of ceftiofur concentrations (µgmL^{-1}) vs area of growth inhibitory zone (mm) was plotted. In regression analysis, determined linear equation was Y = 2.3 x + 11.38 and regression coefficient was (r^2 = 0.9916) (Fig. 3).

Range

Range was measured by the selected reference substance concentrations of the calibration curve and confirmed by linearity, precision, and accuracy of the method.

Precision

The relative standard deviation (RSD) for different precision parameters i.e. repeatability and intermediate precision were calculated. The repeatability of XCEFT powder for injection was determined in six replicates on the same day by the same analyst (Table IV). The intermediate precision was calculated by repeating the analysis on two distinct days (inter day) and between different analysts (inter analyst) (Table V).

Accuracy

Accuracy was measured at 80%, 100%, and 120% of the nominal analytical concentration for the microbiological assay method. The calculated mean accuracy was 101.08%, with an RSD of 0.28% indicating that the approach can accurately determine ceftiofur sodium concentrations in the 80–120% range (Table VI).

Robustness

Table 5: Intermediate precision evaluation for XCEFT powder for injection by bioassay.

Precision	Experimental % potency	Mean % potency	RSD (%)
Inter-day Precision			
Day 1	98.81	99.57	0.81
	99.59		
Day 2	100.69		
	99.20		
Inter-analyst Precision			
Analyst 1	101.06	101.05	0.43
	100.44		
Analyst 2	101.39		
	101.32		

Table 6: Accuracy for XCEFT powder for injection by bioassay

Hypothetical % potency	Experimental % potency	Mean % potency	Accuracy (%)	RSD (%)
	81.75			
80	81.26	81.10		
	80.28			
	100.69			
100	102.07	101.05	101.08	0.28
	100.39			
	120.78			
120	120.36	120.97		
	121.76			

It was determined after examining the same material under various settings. The buffer used for standard dilution, inoculum concentration and various microbial strains were all taken into account. To investigate the resilience, some test settings were altered, including the solvent used for the standard and sample dilution (phosphate buffer pH 7.0), inoculum concentration (3.0%), and incubation temperature (30°C). When the experimental settings were changed to the required specifications, no significant variations in potencies were observed, as shown in Table VII.

Discussion

The development and validation of analytical method for the potency estimation has received considerable attention from regulatory bodies because of their importance in pharmaceutical analysis. 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 complexity, sample quantity, qualitative or quantitative purpose of the method, and the availability of equipment and literature.

In various pharmacopoeias and literatures, HPLC methods and other chemical techniques have been developed and used for potency estimation of ceftiofur sodium in pharmaceutical products. In this case a bioassay method was proposed as a suitable method for estimation of potency of ceftiofur in powder for injection. Antibiotic potency can be determined by comparing the inhibition of growth of a susceptible microbe induced by known quantities of the antibiotic under investigation and their related reference substances. For assessing the ceftiofur sodium content in dry powder injectable pharmaceutical dosage forms, a two-level (2×2) factorial microbiological assay was proposed7.

In the literature studies, there is an established microbiological assay technique by three level (3×3) cylindrical plate method for evaluating ceftiofur sodium activity against *Micrococcus luteus* ATCC 10240.[8] Souza et al. 2007, confirmed the antibacterial activity of ceftiofur sodium against *Micrococcus luteus* ATCC 10240 on Grove-Randall's culture medium (Diffco).[8] In proposed study, microbiological assay of ceftiofur sodium was performed on 09 bacterial strains for their reaction and susceptibility. Few of them show positive response i.e. susceptibility against ceftiofur sodium but due to its ability to form sharp edge zone of

Table 7: Factors studied in the robustness.

Factors	Parameters	Mean % potency	RSD (%)
		101.62	
Solvent	Phosphate buffer pH 7.0	100.42	0.60
		101.20	
		101.39	
Inoculum concentration	3.0%	100.79	0.50
		100.39	
		100.69	
Incubation temperature	30 °C	99.17	0.78
		99.61	

inhibition on Antibiotic assay medium B, *B. subtilis* MTCC-441 was chosen as the most appropriate test organism.

On the basis of clear and sharp edge zone of inhibition, concentration of inoculum should be validated for quantification of an antibiotic throughout a microbial bioassay.[14] Experiments were carried out to determine the importance of inoculum concentration while all other conditions remains constant, as it is well known that inoculum concentration affects the size of the resulting zone.[14,19] Petri plate containing media with high concentration of test organism produce cloudy growth and no antimicrobial action of antibiotic while low inoculum concentration of test organism produce a light or immeasurable zone. Therefore it is necessary to optimize inoculum concentration for microbiological assay. The inoculum concentration of *M. luteus* ATCC 10240 was chosen as 1.0% for the development of ceftiofur sodium bio assay.[8] In proposed study different inoculums concentrations of *B. subtilis* MTCC-441 i.e. 0.5%, 1.0%, 1.5%, 2.0%, and 3.0% were tested and selected optimize inoculum concentration was 2.0% for microbial bioassay.

For this experiment, the area of growth inhibition zones was measured using a selected range of concentrations of the reference substance ceftiofur sodium. The chosen concentration was determined by the microbes susceptibility to low concentrations, the size of inhibitory zones at high concentrations, and the linear relationship between the logarithm of concentration and the mean area of the inhibition zone, which was limited by the size of the petri dish. Other authors used a concentration of ceftiofur sodium of 2-8 μgmL^{-1} for method development. In our study, different concentrations i.e., 1.0, 2.0, 4.0, 5.0 and 10 μgmL^{-1} of reference substance of ceftiofur sodium were tested against selected test microorganism and the high and low concentration of ceftiofur sodium reference substance were chosen as 1.0 µgmL-1 and 4.0 μgmL^{-1} for potency estimation. A good linearity was established in the range of specified concentrations of ceftiofur sodium reference substance by plotting the logarithm of antibiotic concentration (µgmL-1) vs mean diameter of inhibition zone (in mm) (Fig. 3). The representative linear equation for ceftiofur sodium was $Y = 2.3 x + 11.38$ and the regression coefficient ($r^2 = 0.9916$) obtained was very significant for this approach.

Relative Standard Deviation was used to express precision. The repeatability of the sample was determined by analysing it several times on the same day; the mean ceftiofur sodium content in XCEFT powder for injection was 101.79%, with an RSD value of 1.07%. The mean ceftiofur sodium content for interday precision assay was 99.57% with an RSD of 0.81% and the mean content between analysts was 101.05% with an RSD of 0.43%. Accuracy was measured at 80%, 100%, and 120% of the nominal analytical concentration for the microbiological assay method with an RSD of 0.28% and the calculated mean accuracy was 101.08%.

The performed validation and result obtained in this study were satisfactory and proven that bioassay is a good option for pharmaceutical analysis of ceftiofur sodium in powder for injection. It is a useful analytical tool when used in addition to or instead of the physicochemical approach.

Conclusion

For ascertaining the quality of pharmaceutical products, it is mandatory to utilize validated and authenticated analytical methods. HPLC methods for estimating the potency of ceftiofur sodium have been developed in the various pharmacopoeias. However, there is a genuine microbiological method for estimating ceftiofur sodium potency that has not been described in any pharmacopoeias. According to literature survey, microbiological assay method have been developed for ceftiofur sodium against test organisms *M. luteus* ATCC 10240. The purpose of the proposed experimental investigation was to design and validate a bioassay method for estimating ceftiofur sodium potency. The choice and optimization of the microbial assay was performed through the use of various conditions and *B. subtilis* MTCC-441 was found to be the most susceptible organism against ceftiofur. Several parameters including buffer pH, inoculum concentration and standard solution concentration were studied using the test organism *B. subtilis* MTCC-441 against ceftiofur sodium. The potency of ceftiofur sodium in XCEFT powder for injection sample was estimated as 101.79% through bioassay. The results demonstrated that the proposed microbiological assay method for estimating the potency of ceftiofur sodium in pharmaceutical products is accurate, with the obtained results confirming its good accuracy, robustness, precision and significant linearity of response. Therefore, the proposed bioassay method can be useful for the quality control of ceftiofur sodium in the studied formulation.

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