

The Selection of Bacterial Strain and Inoculum Concentration in Microbial Bioassay for the Development of Cefixime Potency Test in Pharmaceutical Preparations

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ABSTRACT

Cefixime is one the antibiotic that use widely in the clinical setting. Microbiological bioassay is an essential step to ensure the efficacy of the antibiotic. Several factors including bacterial strain, inoculum concentration, and solvent, pH, reference concentration, storage time of antibiotic standard influenced the zone inhibition of the bioassay. This study was aimed to optimize two factors from microorganism aspect for the development antibiotic potency test of cefixime in pharmaceutical preparation. Cylinder cup method was used for this microbiological assay. Our results revealed that *Escherichia coli* ATCC 10536 with the concentration of 1.0% was the optimum bacterial strains for cefixime reference standard. In conclusion, the optimizing both factors from the test microorganism could be helpful to develop the appropriate method for microbial bioassay of Cefixime in pharmaceutical preparations.

Keywords: Antibiotic, cefixime, potency test, bacterial, inoculum concentration, *Escherichia coli*.

Introduction

Cefixime, a third generation of cephalosporin antibiotic, is used widely to treat several bacterial infections such as uncomplicated urinary tract infection,

bronchitis chronic, and uncomplicated gonorrhoea (Brogden et al., 1989). It is synthetic a broad-spectrum antibiotic that active against Gram-negative and Gram-positive bacteria. Cefixime inhibits

bacterial growth by inhibit cell-wall synthesis (Matsumoto et al., 2001). Cefixime is the antibiotic with molecular formula $C_{16}H_{15}N_5O_7S_2$ and a molecular weight of 453.3 g/mol (Roche, 1989).

The accurate evaluation of antibiotics' potency and bioactivity is crucial for ensuring the efficacy and its safety use. A slight variation in the concentration of the active ingredient in antimicrobial preparations may have an effect on their actual activity. Therefore, the measurement of the active pharmaceutical ingredient (API) in antimicrobial preparations is crucial (Hewwit, 2012). It is known that these chemicals at extremely low concentrations harm or partially inhibit pathogens (Denyer, 2008).

Antibiotic potency can be measured using chemical and biological techniques. The microbiological bioassay for determining the potency of Cefixime has not yet been published in any monograph. The biological method is the most practical way to measure antibiotic potency (Cazadey and Salgado, 2011). Determining antibiotic potency is crucial for the quality control and quality assurance of pharmaceutical preparations; therefore, it is required to create practical and cost-effective procedures that may be used in the validation and dosing of pharmaceuticals (Yamamoto and Pinto, 2020). Antibiotics can be evaluated for their active components, biological activity, and stability via microbiological bioassay. Any minor alteration in the antibiotic molecule could change the biological

activity. However, the chemical assay such as HPLC cannot directly detect or reflect the bioactivity (Dafale et al., 2015). As a result, microbiological analysis is extremely important for solving issues regarding potential changes in antimicrobial potency and their preparations.

Before developing the validated method of antibiotic potency, several factors are needed to establish, including the test of microorganism and inoculum concentration from the aspect of microorganism, the solvent of antibiotic, the pH of the certain solvent, and the duration of storage time stock solution from the aspect of the antibiotic standard (United State Pharmacopeia, 2010). Those factors must be considered in determining a potential antibiotic preparation using the cylinder cup method due to can affect the inhibition zone produced against the test bacteria. On the other hand, the clarity of zone inhibition will be also affected by those factors, which could affect the measurement of the appropriate zone inhibition. This study is aimed to optimize two factors from microorganism aspect including the proper of bacterial strains and the optimal inoculum concentration.

Research Method

Chemical and Reagents

United States Pharmacopoeia (USP) reference standard of Cefixime was purchased from Sigma Aldrich. Cefixime standard was dissolved in sterile phosphate buffer pH 7.0.

Analytical grade of chemicals and reagents was used in this study.

Preparation of Microbiological Media

Dehydrated media of antibiotic assay medium no. 1 was used as the bioassay medium. Medium were dissolved in the deionized water and pH was adjusted as per manufacturers instruction. Then, medium was sterilized for 15 min at 121°C in the autoclave.

Optimization of Bacterial Strains

Different Gram-positive and negative bacteria were used for the microbiological assay. Several microbial cultures were purchased from American Type Culture Collection (ATCC, USA) including *Escherichia coli* (ATCC-10536), *Staphylococcus aureus* (ATCC-29213), *Pseudomonas aeruginosa* (ATCC-27853) and *Bacillus cereus* (ATCC-11778). In this study, all bacterial strains were tested with cefixime standard solution with five different concentrations with a ratio of 1:1.25 based on the USP recommendation. Then, 5 µg/mL as the median dose or reference concentration "S3". All plates were incubated at 37°C for 24 hours and applied for further optimization.

Optimization of Inoculum Concentration

The stock of selected microbial strain was sub-cultured on the nutrient agar media. Then, the media was incubated for 24 hr at 37°C for the growth of bacteria. Microorganism on the agar media were washed with 3 mL of sterilized saline solution (0.9%)

followed by dilution which showed 25% light transmission at 530 nm. Then, six inoculum concentration were prepared in a volume of 1 mL with concentration of 0.5; 1.0; 1.5; 2.0; 2.5; and 3%, respectively. Furthermore, 1 mL of the prepared inoculum was poured in the petridish with the agar medium using pour plate technique. During the study, new cultures were used for all experiments. In this experiment, 5 µg/mL was selected as the concentration for cefixime standard. All plates were incubated at 37°C for 24 hours.

Results and Discussion

A literature review revealed the use chemical techniques like spectrophotometry for determining the potency of Cefixime in pharmaceutical preparations (Azmi et al., 2013). However, a microbiological assay to determine the potency of Cefixime was not available in any pharmacopoeia. Although chemical method is a rapid technique to assess the potency of an antibiotic, it is incapable of determining bioactivity. However, the microbiological assay measures both the potency and bioactivity of antibiotics. In particular, a bioassay can be performed to determine the effective dose against bacteria resistant to antibiotics. For further study, we propose to create a microbiological bioassay as an appropriate and straightforward technique for measuring Cefixime in pharmaceutical preparations.

The microbiological assay measures the actual effect of antibiotics on a biological system and is used to

provide more accurate and specific evaluations of potency than chemical procedures. The bioassay methods used for potency measurement are the most critical factors in obtaining reproducible and reliable data required for quality control of the pharmaceutical product (Shah et al., 2000). Microbiological bioassay is beneficial due to the similarity between the parameters assessed by these techniques and the characteristics of the drug being evaluated. Thus, impurities and associated compounds do not interfere with the analytical method's precision. Therefore, the microbiological bioassay

remains the standard for addressing doubts regarding probable activity loss of antibiotics (Dafale et al., 2016).

The selection criteria of the bacterial strain were based on the response of the bacteria to cefixime in the form of sensitivity, susceptibility and the clarity of zone inhibition (Dafale et al., 2015). As shown in **Table 1**, the results showed that *S. aureus*, *P. aeruginosa* and *B. cereus* did not respond to cefixime. In contrast, only *E. coli* as a bacterial strain responded against cefixime (**Figure 1B**). As a result, the *E. coli* (ATCC-10536) strain was selected for further analysis in this study.

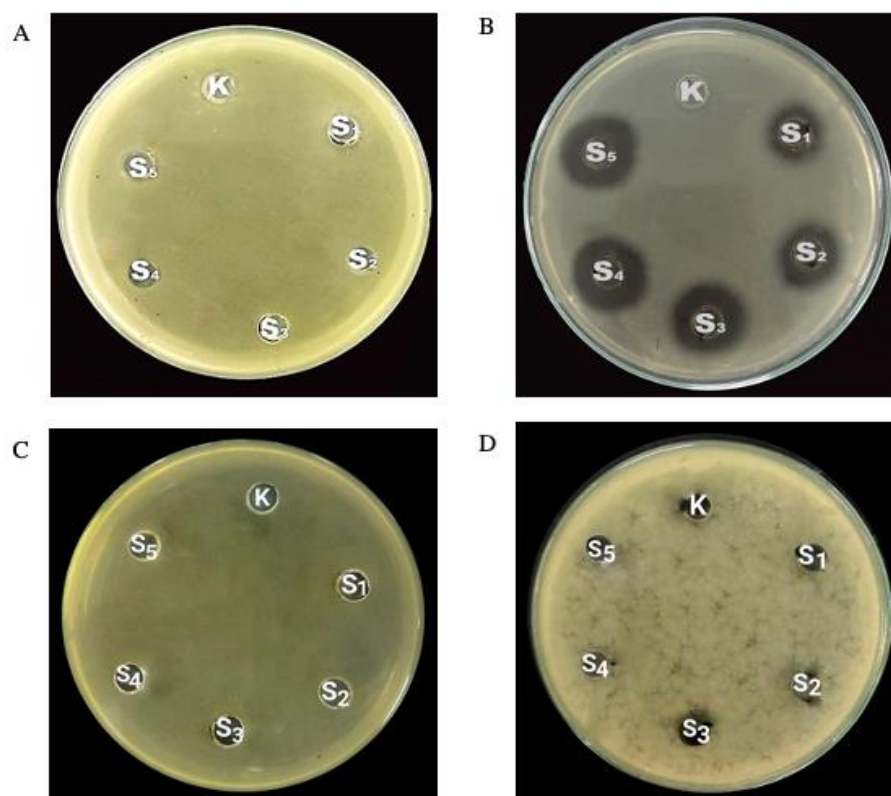


Figure 1. The effect of Cefixime in several bacterial strains at different concentration. *S. aureus* ATCC-29213 (A), *E. coli* ATCC-10536 (B), *P. aeruginosa* ATCC-27853 (C), and *B. cereus* ATCC-11778 (D)

Table 1. Response of several bacterial strains to Cefixime standard at different concentration

Bacterial strains	Code	Concentration of standard Cefixime ($\mu\text{g/mL}$)	Inhibition zone diameter (mm)			Mean (mm)
			I	II	III	
<i>Staphylococcus aureus</i> (ATCC-29213)	K	Solvent	-	-	-	-
	S1	3.2	-	-	-	-
	S2	4.0	-	-	-	-
	S3	5.0	-	-	-	-
	S4	6.2	-	-	-	-
	S5	7.8	-	-	-	-
<i>Escherichia coli</i> (ATCC-10536)	K	Solvent	-	-	-	-
	S1	3.2	12.6	13.1	12.8	12.8
	S2	4.0	13.1	13.4	13.6	13.4
	S3	5.0	15.2	14.7	13.8	14.5
	S4	6.2	18.6	14.5	16.9	16.7
	S5	7.8	19.3	18.9	18.6	18.9
<i>Pseudomonas aeruginosa</i> (ATCC-27853)	K	Solvent	-	-	-	-
	S1	3.2	-	-	-	-
	S2	4.0	-	-	-	-
	S3	5.0	-	-	-	-
	S4	6.2	-	-	-	-
	S5	7.8	-	-	-	-
<i>Bacillus cereus</i> (ATCC-11778)	K	Solvent	-	-	-	-
	S1	3.2	-	-	-	-
	S2	4.0	-	-	-	-
	S3	5.0	-	-	-	-
	S4	6.2	-	-	-	-
	S5	7.8	-	-	-	-

Table 2. Response of *E. coli* to Cefixime (5 $\mu\text{g/mL}$) at different inoculum concentration

Solvent	Inoculum concentration (%)	Inhibition zone diameter (mm)			Mean (mm)	SD	RSD (%)
		Replication					
		I	II	III			
Phosphate buffer pH 7.0	0.5	21.2	21.5	21.1	21.267	0.208	1.0
	1	20.52	20.3	20.2	20.333	0.153	0.8
	1.5	20.1	19.9	19.6	19.867	0.252	1.3
	2.0	19.4	18.9	18.7	19.000	0.360	1.9
	2.5	18.3	18.1	17.9	18.100	0.200	1.1
	3.0	17.9	18.3	17.0	17.967	0.306	1.7

Variation in the number of microorganisms led to differences in the diameter of the inhibition zone. While other variables were constant, this experiment was conducted to establish

how crucial the concentration of the test organism might have been in the current system. The optimal inoculum concentration was determined using zone diameter, edge sharpness, and low

RSD. A small zone diameter was seen with a high inoculum concentration, but a diluted inoculum concentration produced a bigger zone diameter and weak growth. The optimal concentration of inoculum should range between these profiles. In the current experiment, six different inoculum concentrations, including 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, and 3.0%, were evaluated and their effects on the diameter of the zone of inhibition were identified (Table 2 and Figure 2). The optimal *E. coli* ATCC-10536 inoculum concentration for microbial bioassay was determined to be 1%.

To measure an antibiotic by a microbiological bioassay, the inoculum concentration must be confirmed, exhibiting a distinct antibiotic zone of inhibition (Dafale et al., 2013). An effect of inoculum concentration on resultant zone size is widely known. This study evaluated how crucial the inoculum concentration could be when all other variables remain constant

(Dafale et al., 2013; Hewwit, 2003). Different zone diameters are the result of a wide range of microorganism concentrations. A high inoculum concentration of the test bacteria resulted in a small zone diameter and a cloudy growth stage. In contrast, a low inoculum concentration resulted in a broad zone diameter and a light growth stage. Consequently, optimizing the inoculum concentration is required for a bioassay.

Conclusion

For the development of antibiotic potency test of cefixime in pharmaceutical preparation, our study suggested that *E. coli* ATCC 10536 can be used as the bacterial strain with the concentration of 1.0% as the optimal inoculum concentration. For further study, several factors from the aspect of antibiotic such as the proper solvent, pH, and duration of storage time should be optimized.

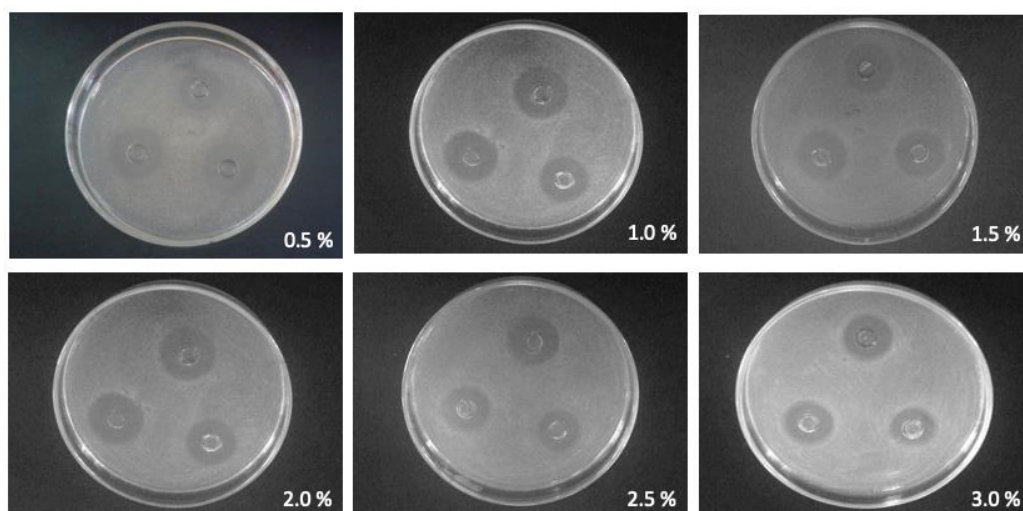


Figure. 2. The effect of Cefixime at different concentration of *E. coli* inoculum

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