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# Comparative Study by *In Vitro* Method of Antimicrobial Activity in Different Commercial Antibiotics Cefoperazone-Sulbactam Products

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**ABSTRACT**

*The antimicrobial activity of Cefoperazone-Sulbactam was studied by microbiological assays in order to determine their potency by diffusion gel method. Bacillus subtilis ATCC 6633 was established as a microbiology model, and comparison study of antibiotic samples using USP standard, generic and originals was executed. Procedures were established in the laboratory, capable of handling experiential test for assaying various execution biomass conditions such as bacteria growing, incubation times and inhibition zones as a response to various concentrations of antibiotic. Also, the bio-assay`s methodology was validated. This study revealed all antibiotic samples behave in similar ways, through in vitro methods. We therefore conclude that all of the samples are pharmaceutical equivalents and the products can be used in the antimicrobial therapy.*

**Keywords:** Cefoperazone-Sulbactam; Antimicrobial activity; Diffusion gel

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## INTRODUCTION

Cephalosporin is the largest and most diverse family of beta-lactam antibiotics. Cephalosporin is indicated for the prophylaxis and treatment of infections caused by bacteria susceptible to this particular form of antibiotic. They are structurally and pharmacologically related to the penicillin. Cephalosporin has a betalactam ring structure, infused to a 6-membered dihydrothiazine ring, thus forming the cephem nucleus and interferes with bacterial cell wall synthesis. Cephalosporin distrusts the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan

layer is important for cell wall structural integrity. Cephalosporins, since its introduction in 1960, it has been widely used and have shown low toxicity rates and favorable Pharmacokinetic profiles. They are usually classified in "generations", based on its spectrum of activity, they have been classified in 3 generations and in 1997 was approved a fourth generation. Their antimicrobial effect by interfering with the synthesis of the Peptidoglycan, the greater proportion in the microbial cell wall component, which provides rigidity to the bacterium. As part of normal cell growth and division, pentapeptide Peptidoglycan components, are initially synthesized in the cytoplasm, transported across the cytoplasmic membrane and inserted into the existing Peptidoglycan by enzymes such as carboxypeptidases, endopeptidases and transpeptidasas. These enzymes that are found in the cytoplasmic membrane are called (PBLS) penicillin binding proteins and are the targets of action of beta-lactam drugs [1]. The sodium Cefoperazone, is an antibiotic third generation cefalosporinico, this antibiotic is active in Vitro against a wide range of aerobic and anaerobic, Gram-positive and Gram-negative pathogenic microorganisms. The bactericidal action exercised by this antibiotic is the result of inhibition of the synthesis of cell wall of the bacteria. The cefoperazone, possesses a high degree of stability in the presence of beta-lactamases that are produced by many gram-negative bacteria. (CEFOBID ®) [2]. Sulbactam is a derivative of the core of penicillin, a beta-lactamase inhibitor. It is used to increase the bacterial spectrum of Penicillins and cephalosporins against producing penicillinase and beta-lactamase is produced by micro-organisms such as *Staphylococcus aureus* and *Moraxella catarrhalis* that are resistant to ampicillin. Sulbactam irreversibly inhibits the beta-lactamase. However, its interaction with cefalosporinasa (AmpC) is not possible. It confers protection against bacteria such as *Pseudomonas aeruginosa*, *Citrobacter, Enterobacter*, and *Serratia*, which often express this gene. Sulbactam is combined to form: Cefoperazone-Sulbactam (Sulperazone) and ampicillin-Sulbactam (Sultamicilin) [3]. The objective of this research was to determine the antimicrobial activity of different commercial samples of Cefoperazone-Sulbactam antibiotic intravenous administration through in-Vitro methods through the development and subsequent validation of an analytical method.

## MATERIALS AND METHODS

### Microorganisms

The microorganism were provided by Pontificia Universidad Javeriana. *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 29737, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12224, *Pseudomonas aeruginosa* ATCC 9027, *Pseudomonas aeruginosa* ATCC 25619, *Micrococcus luteus* ATCC 9341, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 10536, *Escherichia coli* ATCC 54127, *Klebsiella pneumoniae* ATCC 10031.

### Culture Media and Reagents

Agar Mueller Hinton, MERCK, conservation solution, Agar antibiotic No 1, Agar Baird Parker MERCK, Agar ENDO, MERCK, standards USP of Cefoperazone and Sulbactam, commercial samples of Cefoperazone-Sulbactam purchased in pharmacies, generic samples provided by Vitalis S.A. Potassium phosphate bibasico pure pH 8.0 MERCK, sodium phosphate anhydrous monobasic pH 6.0, MERCK, sodium hydroxide and hydrochloric acid MERCK.

**Bioassay Inoculum**

The bacterial suspension was standardized following the CLSI guidelines and was grown in Mueller-Hinton broth (HiMedia) for 18-24 hrs, followed by the matching of bacterial suspension to the turbidity equivalent to 0.5 McFarland solution (1-2×$10^8$ CFU/mL) with the addition of sterile saline [4]. Then, all microorganisms were activated in agar Mueller Hinton to obtain isolated colonies and incubated at 37°C for 24 h with the exception of *Bacillus subtilis* ATCC 6633 with a time of 8 h [5,6], incubation with isolated colonies is made massive planting on agar Mueller Hinton. From mass planting, is made a suspension of microorganisms which conforms to the 25 %T at 600 nm for the standardization of the inoculum. From massive plating in agar Mueller Hinton, microorganisms were harvested from conservation solution. From this solution concentrated microorganisms with the same solution a suspension of 25% of transmittance measured at 600 nm [6-8].

**Stock Solutions and Dilutions of the Antibiotic**

Cefoperazone-Sulbactam, was dissolved in 1000 µg/ml concentration buffer phosphate pH 6.8, 0.45 M, Then was served 5 ml antibiotic agar No 1, which was the average basis, and 5 mL of medium seeds that had been inoculated with microorganism suspension at (25 %T). It was allowed to solidify and boxes were labeled with the name of each microorganism. 6 cylinders of Crystal polystyrene template was placed and was added to Wells 100 µl of each of the concentrations of the antibiotic in study. Boxes were left in predifusion for 30 minutes, and incubated at 37 ºc for all micro-organisms for 24 hours except *Bacillus subtilis* ATCC 6633, the halos of inhibition with gauge were measured for who the incubation time was 8 hours [6-8].

**Difusion Method**

The agar diffusion method was used, incorporating the microorganism suspension into the culture media at a suitable temperatura (Hewitt, 1977) and zone diameters were measured by following Clinical and Laboratory Standards Institute (CLSI) guidelines [9,10]

**Statistical Analysis**

The statistical calculations were carried in an excel program, included in the Microsoft Windows XP package.

## RESULTS

**Determination of Microorganism Model**

*Bacillus subtilis* ATCC 6633 was chosen as a microorganism model or pattern, in accordance compliant with its appropriate sensitivity to the different concentrations of antibiotic used, and a high correlation between inhibition diameters and concentrations used. Halos were well defined, clear and easy to measure; as well as easy handling during preparation and methodology development (Figure 1).

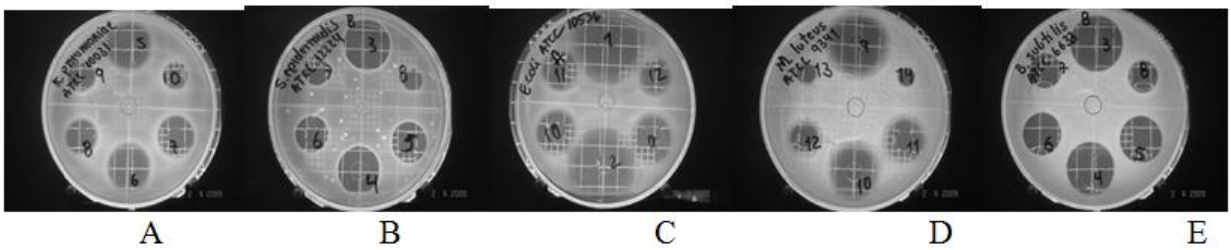


**Figure 1. Responses of microbial models front of Cefoperazone-Sulbactam (A) K.pneumoniae ATCC 10031, (B) St. Epidermidis ATCC 12224 c *E. coli* ATCC 10536 (D) *Micrococcus luteus* ATCC 9341. (E) *Bacillus subtilis* ATCC 6633.**

**Effect of pH in the solutions of Cefoperazone-Sulbactam**

Tests by changing the pH of the medium was realized trying with different pH 5.8, 6.3, 6.8, 7.3 and 7.8; with HCL or NaOH, in order to know the effect of different pHs in antibiotic molecule. Test were performed and was done directly in the culture medium, was carried out adjustment by least squares equation of the straight line of all evaluated pH (Table 1). Was obtained and did not find significant variation. pH 6.8 was chosen to carry out the tests, due to its intercept and according to [11] at this pH the cephalosporins have better stability.

**Table 1. Determination of the effect of the pH of the medium to Cefoperazone-Sulbactam buffer phosphates**

| Concentration | | Average halos (mm) | | | | |
|---|---|---|---|---|---|---|
| (µg/ml) | Ln | pH 5.8 | pH 6.3 | pH 6.8 | pH 7.3 | pH 7.8 |
| 100 | 4.6051702 | 26.19 | 25.05 | 25.27 | 25.87 | 26.01 |
| 50 | 3.912023 | 24.26 | 23.32 | 23.42 | 23.9 | 23.96 |
| 25 | 3.2188758 | 22.44 | 21.23 | 21.36 | 21.45 | 22.01 |
| 12.5 | 2.5257286 | 21 | 19.09 | 18.64 | 19.76 | 20.71 |
| 6.25 | 1.8325815 | 18.54 | 16.42 | 16.96 | 18.06 | 17.97 |
| 3.125 | 1.1394343 | 16.04 | 15 | 14.71 | 18.85 | 16.13 |

**Range of Concentration**

The organism model and antibiotic concentration for an adequate response, was defined; considered and inhibitory size response was between to 10 – 25 mm, the twelve dilutions and concentration were made; two ranges of concentrations that met this condition, C5-C9, C6-C10, the concentration range to C5-C9 was chosen based on correlation coefficient close to 1.

**Validation the Analytic Methodology**

Parameters of linearity, Precision in terms of repeatability and reproducibility were evaluated and so the statistical analyses were performed (Table 2).

Linearity. Was defined as proportionality between analyte and its response. These correlations were calculated by adjusting squares, and the use of statistics as t student and probability. Establish tests of hypotheses for the slope, intercept and correlation coefficient, and to demonstrate that the methodology is linear, should reject the null hypothesis, which took place at the statisticians calculated.

**Table 2. Determination of level of accuracy, repeatability, and reproducibility for the 1 and 2 analysts.**

| Analyst 1 Average Halos (mm) | | | | | | Analyst 2 Average Halos (mm) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 50 | 25 | 12.5 | 6.25 | 3.125 | 50 | 25 | 12.5 | 6.25 | 3.125 |
| Average | 26.99 | 24.5 | 22.67 | 20.65 | 18.35 | 26.73 | 25.23 | 23.37 | 20.97 | 18.4 |
| D.S | 0.712 | 0.7656 | 1.0733 | 0.7171 | 0.8667 | 0.572 | 0.891 | 0.794 | 0.4515 | 0.7549 |
| C.V | 2.639 | 3.1246 | 4.7345 | 3.4722 | 4.7221 | 2.141 | 3.53 | 3.395 | 2.1535 | 4.1035 |
| Average C.V | | | | | 3.7385 | Average C.V | | | | 3.0646 |

Precision. We checked two levels of precision, repeatability, defined as assay Precision, and reproducibility, as the precision between laboratories, was in this case evaluated by comparing two analysts and intralaboratory. For

repeatability, calculations of average, standard deviation and coefficient of variation among five concentrations taken as range work and three tests were made.

Results of Stability tests (Tables 3-6) were evaluated with the following temperatures: 4°C, 25°C, 37°C, 50°C and at the following times: 6, 12, 24, 48, 72 h, after a time, the trial was conducted in order to determine the action or involvement of the molecule of antibiotic against the different temperatures.

**Table 3. Evaluation of the stability of Cefoperazone-Sulbactam buffer phosphate pH 6.8 0.45 M at 4°C for 0, 6, 12, 24, 48, 72 hours and 7 days.**

| CONCENTRATIONS | | | t0 | t6 h | t12 h | t24 h | t48 h | t72 h | t7 días |
|---|---|---|---|---|---|---|---|---|---|
| | (µg/ml) | Ln Concentratión | | AVERAGE INHIBITION HALOS (mm) | | | | | |
| C1 | 50 | 3.912023 | 24.9 | 20.89 | 25.1 | 23.55 | 26.86 | 23.18 | 25.41 |
| C2 | 25 | 3.2188758 | 21.9 | 19.07 | 22.48 | 21.32 | 23.79 | 21.47 | 23.61 |
| C3 | 12.5 | 2.5257286 | 19.66 | 17.46 | 20.44 | 19.4 | 22.01 | 20.39 | 21.52 |
| C4 | 6.25 | 1.8325815 | 17.05 | 15.72 | 17.92 | 17.25 | 20.04 | 17.44 | 19.08 |
| C5 | 3.125 | 1.1394343 | 15.41 | 14.63 | 15.92 | 15.64 | 18.08 | 16.2 | 16.84 |
| C6 | 1.56 | 0.4446858 | 12.83 | 12.97 | 13.67 | 13.96 | 15.58 | 14.59 | 15.2 |

**Table 4. Evaluation of the stability of Cefoperazone-Sulbactam buffer phosphate pH 6.8 0.45 M at 25°C for 0, 6, 12, 24, 48, 72 hours and 7 days.**

| CONCENTRATIONS | | | t0 | t6 h | t12 h | t24 h | t48 h | t72 h | t7 días |
|---|---|---|---|---|---|---|---|---|---|
| | (µg/ml) | Ln Concentratión | | Average inhibition Halos (mm) | | | | | |
| C1 | 50 | 3.912023 | 24.9 | 21.48 | 24.29 | 23.64 | 23.78 | 23.08 | 23.91 |
| C2 | 25 | 3.2188758 | 21.9 | 18.9 | 21.93 | 21.59 | 21.35 | 21.16 | 22.49 |
| C3 | 12.5 | 2.5257286 | 19.66 | 17.34 | 19.86 | 19.9 | 19.94 | 19.63 | 19.74 |
| C4 | 6.25 | 1.8325815 | 17.05 | 14.98 | 17.65 | 17.98 | 17.68 | 17.21 | 18.16 |
| C5 | 3.125 | 1.1394343 | 15.41 | 12.65 | 15.51 | 16.18 | 15.6 | 15.66 | 16.42 |
| C6 | 1.56 | 0.4446858 | 12.83 | 9.99 | 13.64 | 13.74 | 13.1 | 14.35 | 14.39 |

**Table 5. Evaluation of the stability of Cefoperazone-Sulbactam buffer phosphate pH 6.8 0.45 M at 37°C for 0, 6, 12, 24, 48, 72 hours and 7 days.**

| Concentrations | | | t0 | t6 h | t12 h | T24 h | t48 h | t72 h | t7 días |
|---|---|---|---|---|---|---|---|---|---|
| | (µg/ml) | Ln Concentratión | | Average inhibition halos (mm0 | | | | | |
| C1 | 50 | 3.912023 | 24.9 | 21.36 | 23.84 | 23.06 | 22.83 | 21.48 | 17.64 |
| C2 | 25 | 3.2188758 | 21.9 | 19.5 | 21.71 | 20.78 | 19.16 | 19.53 | 16.41 |
| C3 | 12.5 | 2.5257286 | 19.66 | 18.4 | 18.82 | 18.4 | 17.42 | 17.72 | 14.24 |
| C4 | 6.25 | 1.8325815 | 17.05 | 16.52 | 17.39 | 16.91 | 15.16 | 16.11 | 12.32 |
| C5 | 3.125 | 1.1394343 | 15.41 | 14.32 | 15.41 | 14.31 | 12.92 | 14.09 | 10.61 |
| C6 | 1.56 | 0.4446858 | 12.83 | 12.89 | 13.4 | 12.44 | 10.22 | 13.03 | 9.31 |

**Table 6. Evaluation of the stability of Cefoperazone-Sulbactam buffer phosphate pH 6.8 0.45 M at 50°C for 0, 6, 12, 24, 48, 72 hours and 7 days.**

| Concentrations | | | t0 | t6 h | t12 h | t24 h | t48 h | t72 h | t7 días |
|---|---|---|---|---|---|---|---|---|---|
| | (µg/ml) | Ln Concentratión | | Average inhibition Halos (mm) | | | | | |
| C1 | 50 | 3.912023 | 24.9 | 20 | 21.46 | 17.39 | 15.54 | 10.47 | 9.4 |
| C2 | 25 | 3.2188758 | 21.9 | 18.27 | 18.96 | 16.02 | 13.67 | 9.96 | 9.3 |
| C3 | 12.5 | 2.5257286 | 19.66 | 15.87 | 17.07 | 14.74 | 12.61 | 9.76 | 9.27 |
| C4 | 6.25 | 1.8325815 | 17.05 | 13.24 | 14.83 | 13.22 | 10.8 | 9.32 | 9.21 |
| C5 | 3.125 | 1.1394343 | 15.41 | 11.78 | 13 | 11.25 | 9.96 | 9.05 | 9.14 |
| C6 | 1.56 | 0.4446858 | 12.83 | 9.71 | 10.94 | 9.35 | 9 | | 9.07 |

**Valuation of commercial samples of Cefoperazone-Sulbactam**

Commercial samples of Cefoperazone-Sulbactam was a blind study which evaluated different samples of the innovative antibiotic such as generic, obtaining in this way a total of 8 samples to work. For the assessment of samples and calculating power, was used the model approved by the pharmacopoeia [8].

## DISCUSSION

In both test dilutions of the pattern as a sign they have a theoretical power equally, however, to the graph the results, the resulting lines not are usually superimposed and their real difference in power is based on the vertical distance between the two lines that are parallel, i.e. the vertical difference in the Ln or Log [12].

Ph ideal for maintaining the integrity of the molecule of Cefoperazone-Sulbactam and inhibitory capacity to generate optimal variables of temperature and exposure time must be 6.8 at a concentration of 0.45 M, i.e. with Buffer phosphate Ph 6.8 0.45 M according to Luan et al. [13].

The activity of Cefoperazone-Sulbactam remained significantly through exposure time given that retained the kinetics of the antibiotic favoured by the temperature, as well as highlighting the parallelism of each of the straight lines which determines that he remains the antibiotic response subjected to 4°C. When solutions of Cefoperazone-Sulbactam were subjected to a temperature of 25°C an effective response according to the trend of the points was observed in each exposure time, i.e. there is a negative response regarding the composition of the molecule or significant alterations of the antibiotic to stay this temperature at an exposure time of 7 days. Cefoperazone-Sulbactam solutions subjected to 37°C show a different response with respect to previous temperatures where the points of exposure 6, 12 times and 24 hours do not present significant differences with the antibiotic response and we found activity optima arises to 12o'clock and exposure 48 times, 72 hours and 7 days present a decrease in activity in terms of the response of the antibiotic which determines There is an alteration of the molecule after 24 hours and a degradation directly proportional to the exposure time. Solutions of Cefoperazone-Sulbactam subjected to a temperature of 50°C showed a gradual decline in antibiotic response and a negative response in the different exposure times, the molecule of the antibiotic at that temperature of thermal stress, already does not exert their antimicrobial action. When different samples of Cefoperazone-Sulbactam were compared with the values of the standard USP of the same antibiotic we find that there is no great variation with respect to the correlation coefficient of the different samples, which predicts that the samples comply with the specifications of dose- response of the

antibiotic compared to the standard USP of the antibiotic. When the eight samples of Cefoperazone-Sulbactam data were subjected to the calculations for the determination of the relative potency as compared to the USP standard antibiotics and the power of the same, find that there is no significant difference with respect to the power of the different samples, also indicated that the samples comply with the specifications of relative antibiotic potency. According to USP 39, the calculated percentage of relative potency must be among the (80% and 125%), and specifically to Cefoperazone sodium. It must contain the equivalent of not less than 90% and not more than 120% of amount labeled [14].

## CONCLUSION

The range of established work was 2.5 μg/ml$^{-3}$, 125 μg/ml, and evaluated both generic and innovative products comply with provisions of the USP and can be considered pharmaceutical equivalents.

**Ethical Issues**

Not applicable.

**Conflict of Interest**

There is no conflict of interest to declare.

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