UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
Faculdade de Farmácia
Disciplina de Trabalho de Conclusão de Curso de Farmácia

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MICROBIOLOGICAL ASSAY FOR QUANTITATIVE DETERMINATION OF THE ANTIBIOTIC IMIPENEM IN POWDER FOR INJECTION.

Porto Alegre, junho de 2018

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Trabalho de conclusão de curso de graduação apresentado para obtenção do grau de farmacêutico (a) do curso de Farmácia da Universidade Federal do Rio Grande do Sul.

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Porto Alegre, junho de 2018.

“ O homem que move montanhas, começa carregando pedras pequenas. ”

(Provérbio Chinês)

AGRADECIMENTOS

Agradeço primeiramente a Deus pela oportunidade de ter chegado até aqui, pelo amparo nos momentos difíceis e por enviar Seus anjos na forma de pessoas que tanto me ajudaram a galgar esse importante sonho, por todos os momentos em que me deu forças para seguir firme mesmo quando o mundo parecia contra mim. Em segundo lugar agradeço à minha mãe, por tantos anos de amor, educação e todo o seu sacrifício pessoal para que eu alcançasse meus objetivos, sempre me apoiando e me dando o maior exemplo de força, integridade e humildade que eu tive na vida. Gostaria muito que a senhora estivesse presente na concretização dessa vitória, mas entendo que Deus a quis do lado Dele para uma missão maior; a levarei sempre em meu coração e aqui transmito a ti todo o meu amor e gratidão.

Agradeço também ao meu namorado Carlos por ter sido meu parceiro durante todos esses anos de graduação, por estar do meu lado em todos os momentos, felizes e tristes, por todo amor e cuidado que sempre teve comigo. À sua família, da qual hoje posso dizer que faço parte (Iara, Armando, Jaqueline, William), sempre me dando apoio e incentivo, fundamentais para que eu mantenha a fé. Aos demais familiares (pai, irmão) que apoiaram e torceram por mim mesmo de longe, nada seria possível sem vocês.

À Universidade Federal do Rio Grande do Sul, pelo acolhimento, suporte, estrutura e formação de excelência que me permitiu crescer tanto como profissional quanto como pessoa. Ao meu orientador Prof. Dr. Andreas por toda a paciência, incentivo, disponibilidade e conselhos transmitidos. Por conseguinte, ao Laboratório de Controle de Qualidade Farmacêutico da Faculdade de Farmácia da UFRGS e todos os seus integrantes (alunos, professores, funcionários) por todo o aprendizado que me proporcionaram, bem como a estrutura que permitiu a realização deste trabalho de conclusão de curso, em especial ao IC Leonardo Pezzi por sua colaboração e empenho nos experimentos.

Aos meus amigos e colegas de graduação, em especial ao Felipe Catarino, por toda amizade nesta dura jornada, sempre a tornando tão mais leve a cada dia de intermináveis aulas. Finalmente, minha eterna gratidão à cidade de Porto Alegre e ao estado do Rio Grande do Sul, por terem me recebido tão bem anos atrás e terem se tornado meu lar, onde me tornei uma pessoa melhor e conheci pessoas especiais.

APRESENTAÇÃO

O presente artigo foi escrito segundo as normas da revista Current Pharmaceutical Analysis, que constam em anexo. O mesmo consiste nos seguintes tópicos: Introdução, Material e Métodos, Resultados e Discussão, Conclusões e Referências. Toda a parte experimental foi realizada no Laboratório de Controle de Qualidade Farmacêutico, situado na Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul.

SUMÁRIO

Microbiological Assay for Quantitative Determination of the Antibiotic Imipenem in Powder for Injection.

Running title: Microbiological Assay for Quantitation of Imipenem.

RESUMO

O presente trabalho aborda o desenvolvimento e validação de um método microbiológico utilizando o ensaio de cilindros em placas, para a determinação quantitativa do antibiótico imipenem na forma farmacêutica de pó para injeção. O objetivo do trabalho envolveu o estudo analítico de desenvolvimento de uma metodologia alternativa efetiva e de baixo-custo aos métodos físico-químicos já descritos, e aplicável na rotina de controle de qualidade deste antibiótico. Na etapa de desenvolvimento do método, foram otimizadas as condições analíticas, tais como: escolha do microrganismo teste, concentração do inóculo e faixa de concentração das soluções amostra e padrão, permitindo a adequada medição dos halos de inibição. Empregou-se *Staphylococcus epidermidis* ATCC 12228 como microrganismo teste, concentração do inóculo de 2%, e faixa de concentração de análise entre 0,5-2,0 µg/mL. O protocolo de validação seguiu as guias oficiais e os parâmetros avaliados foram linearidade, precisão (precisão intermediária e repetibilidade) e exatidão. No estudo da linearidade, todas as curvas padrão obtidas tiveram coeficiente de correlação superior a 0,999 e a ANOVA confirmou que não houve desvios de linearidade (p-valor < 0,05). O método mostrou-se preciso, com valores de DPR (%) na faixa de 0,28-0,64 (intra-dia), e de 2,49 (inter dias). Os experimentos foram realizados em 3 dias, sendo 3 ensaios com 8 placas por dia. O teor médio de imipenem foi de 101,05 %. A exatidão foi avaliada pelo teste de recuperação, com percentual recuperado de 101,70 - 107,90%, sendo considerado satisfatório. Dessa forma, o método proposto foi considerado validado e adequado à determinação quantitativa do imipenem em pó para injeção, sendo uma alternativa no controle de qualidade deste antibiótico.

Palavras-chave: imipenem, ensaio microbiológico, determinação quantitativa, validação, controle de qualidade.

ABSTRACT

This work describes the development and validation of a microbiological method using the cylinder-plate assay for quantitative determination of imipenem in powder for injection. The aim was to obtain a low-cost and suitable methodology which can be an alternative to physicochemical techniques already described, contributing for the quality control of this antibiotic. Firstly, the analytical conditions were optimized, such as the choice of the test microorganism, inoculum concentration and best range of sample and standard concentrations, in a way that provides the adequate measurement of the inhibition halos. *Staphylococcus epidermidis* ATCC 12228 was selected as test microorganism, 2.0 % of inoculum concentration, and the analytical concentration ranging 0.5-2.0 μg mL^{-1}. The validation protocol followed the official guidelines, and the parameters evaluated were linearity, precision (intermediate precision and repeatability) and accuracy. All standard curves obtained in linearity study showed r values higher than 0.999, and ANOVA confirmed that were no deviation from linearity (p-value < 0.05). The method also proved to be precise with RSD (relative standard deviation) values ranging 0.28-0.64 for repeatability and 2.49 for intermediate precision. It was performed three days of experiments, being three assays of eight plates a day. The drug mean content was 101.05%. Accuracy was assessed by recovery test, with standard recovery percentage of 101.70-107.90% (mean recovery = 104.86%), which was considered satisfactory. Therefore, the proposed microbiological method was considered validated and suitable for application in quantitative determination of this drug, being useful for quality control routine.

Keywords: imipenem, microbiological assay, quantitative determination, validation, quality control.

1. Introduction

Imipenem (Fig.1), or chemically (5R,6S)-3-[2-(aminomethylideneamino) ethylsulfanyl]-6-[(1R)-1-hydroxyethyl]-7-oxo-1-azabicyclo [3.2.0] hept-2-ene-2-carboxylic acid, is a broad spectrum beta-lactam antibiotic belonging to the subgroup of carbapenems, which are drugs structurally composed of a pentacyclic thiazolidinic ring attached to the nitrogen of the beta-lactam ring by a tetrahedral carbon atom [1]. Derived from thienamycin, imipenem is the oldest carbapenems available for clinical use, approved in 1986 in the USA [2]. It has activity against most of the Gram-positive and Gram-negative, aerobic and anaerobic bacteria [1], including those beta-lactamase-producing bacterial pathogens. This occurs due the greater stability of pentacyclic ring that is attached to beta-lactam ring against the action of these enzymes [3]. However, drug-resistance has been reported in Gram-negative bacteria since the drug was introduced in the market, for example in some strains of *Pseudomonas aeruginosa* [4].

Currently in Brazil, in addition to imipenem, the only carbapenems marketed for clinical use are meropenem and ertapenem. Doripenem the newest of the group, has recently completed Phase III clinical trials but is marketed only in EUA and Europe [5]. Clinically, imipenem is used to treat several polymicrobial infections, such as pneumonia and complicated gastrointestinal tract infections, bacteremia, and etc. The recommended dose is 2 g/day in adults with normal renal functions [4]. However, imipenem is hydrolyzed by a renal dehydropeptidase (dehydropeptidase I) [3], being inactivated, requiring co-administration of cilastatin, an inhibitor of this enzyme in clinical use. Imipenem and cilastatin are marketed as powder for injection (1: 1 w/w) in doses of 250 and 500 mg, since they are not absorbed orally. Most often they are given intravenously by infusion, but in case of treatment less severe infections, they can be given intramuscularly (in dose of 750 mg) [6]. For convenience, in this paper, the term imipenem will be used to refer to the drug with cilastatin.

Antibiotic drugs can be analytically determined in biological fluids and pharmaceuticals by several methods. Widely described in the literature are physicochemical techniques as high-performance liquid chromatography (HPLC) methods, coupled with several detectors, such as diode array and mass spectrometry. To determine imipenem in biological fluids are found in literature some HPLC methods, by ultraviolet (UV) detection for human plasma [7-8], by diode-array detection for mouse plasma [9] or spectrophotometric and chemometric technique for human urine [10] and so on, some of it for application in pharmacokinetic studies. On the other hand, for pharmaceutical dosage forms, the literature reports derivate spectrophotometric [11-12], and Reverse Phase-UPLC method for quantification of related imipenem impurities in dosage forms for injection [13]. In fact, antibiotics play an increasingly important role in pharmacotherapy because of their wide use in the clinical practice, so it is extremely important to develop alternative methods that allow their analytical determination in existing pharmaceutical forms [14].

Microbiological assays have been widely used to determinate antibiotic drugs along the years [14-16]. These methods are recommended by several pharmacopoeias, like the USA and European Pharmacopoeias [17-19]. Different techniques are described in microbiological assays that are used for antibiotic drugs for determinate antibacterial activity, like paper disk procedures, standard broth microdiluition assay, and cylinder-plate assay. The latter, besides the evaluation of the inhibitory effect of the antibiotic on the growth of the test microorganisms [14], makes possible its quantitative determination by means of logarithmic relation between the drug concentration and antimicrobial potency.

Therefore, microbiological assays can be an alternative to the physicochemical methods like HPLC, not requiring sophisticated equipment or toxic solvents, and being viable to use even in small laboratories. For carbapenems, two microbiological cylinder-plate assays are described for meropenem and doripenem, both developed to determine the drugs in powder for

injections [14,20] There is no microbiological cylinder-plate assay described in official codes or scientific literature for quantitative determination of imipenem in pharmaceutical dosage form. Thus, the present manuscript reports the development and validation of microbiological assay to determinate imipenem in powder for injection, applying the cylinder-plate method.

2. Material and Methods

2.1. Chemicals

Imipenem reference standard (85.5%) was obtained from Sigma-Aldrich (St. Louis, Missouri, EUA). Powder for injection containing 500 mg of Imipenem monohydrate and 500 mg of Cilastatin sodium was purchased from ABL Antibiotics (Cosmópolis, SP, Brazil). For microbiological assay, Difco™ Antibiotic Media number 11 obtained from Interlab® (São Paulo, SP, Brazil), and Grove Randall number 1 agar from Merck (Darmstadt, Germany) were used. All solutions were prepared in distilled water and sterilized before each experiment. Analytical grade sodium chloride of the Synth LTDA (São Paulo, SP, Brazil) was used for preparation of inoculum suspension.

2.2. Microbiological assay

2.2.1. Microorganism and inoculum

Staphylococcus epidermidis ATCC 12228 was chosen as microorganism test for development of this study. The strains were cultivated on slant preparation with number 1 agar and kept in freezer. One day before the assay, the microorganism was inoculated to another slant preparation with the same agar that was kept in incubator at 35° C for 24 hrs. The inoculum suspension was prepared using NaCl 0.9% solution sterile, which was standardized with 25 ± 2% of transmittance at 580 nm, using a spectrophotometer Analyser® Model 800 (São Paulo, SP, Brazil). NaCl 0.9% solution was used as blank of absorption. Portions of this inoculum

suspension were inoculated into erlenmeyer flasks containing number 11 agar in order to obtain a concentration of 2% inoculum, being kept at 47 ± 2° C. Then, 6 mL of this inoculum (2%) were applied over the base layer (composed by 20 mL of number 11 agar) in the plates.

2.2.2. Standard and sample solutions

Imipenem standard solution was prepared for each experiment. Sterile distilled water was used as solvent for drug solubilization and subsequent dilutions. The drug (10 mg) was accurately weighed and transferred to 100 mL volumetric flask, and distilled water was added to make up to volume and give the final concentration of the stock solution. This stock solution (imipenem 100 µg/mL) was used during the experiment. For sample solution, the same protocol was applied starting from an amount equivalent to 10 mg of imipenem powder for injection. Both stock solutions (100 µg/mL) were used in the subsequent dilutions.

2.2.3. Cylinder-plate assay

The 3 x 3 design was performed with 8 plates for assay, following the procedure recommended by official reference [18]. The 100 x 20 mm petri dishes were filled with number 11 agar in two separate layers: the base layer and the surface layer. The base layer was composed by 20 mL of number 11 agar and the surface layer was composed by 6 mL of inoculum (2%). Six 8 mm cylinders were distributed over the surface layer in each plate, for subsequent application of the drug-containing solutions.

These cylinders were filled with 200 µL of standard solution or sample solution in three different concentrations of each, and then, the plates were incubated in dry air stove at 37 °C for 18 hours. Inhibition halos were measured using a digital caliper Starret® and the data collected were analyzed for antimicrobial potency calculations, by correlating diameter of inhibition halos and log of drug concentration.

2.2.4. Preliminary assays

Two concentrations were tested for inoculum suspension (1% and 2%) in a preliminary assay with 20 plates, being 10 plates with each concentration of inoculum. At the same preliminary experiment, two ranges of concentrations for standard and sample solutions were tested: 1; 2 and 4μg/mL and 2; 4 and 8 μg/mL, which was prepared from the stock solutions. In the plates with 1% of inoculum concentration, the two ranges of dilutions for standard and sample solutions were tested (5 plates for each group). The same procedure was done for the plates with 2% inoculum concentration. The results were compared to evaluate the microorganism growth and inhibition halo measurements.

Another preliminary experiment was performed from observation of previous results, using 2% of inoculum concentration and testing three new concentrations for standard and sample solutions: 0.5; 1 and 2 μg/mL. Here, the focus was the inhibition halo sizes, which must to be adequate for practical measurement and to avoid possible interferences.

2.2.5. Method validation

2.2.5.1. Linearity and Precision (repeatability and intermediate precision)

To evaluate linearity, three concentrations were prepared from the stock solutions of imipenem standard. The stock solution was diluted in volumetric flasks (10 mL) with sterile distilled water and the final concentrations obtained was 0.5; 1 and 2 μg/mL. Linearity was assessed by constructing nine standard curves in three days of assay, being three curves a day, and each curve considered the results from eight plates. The analysis of linear regression was performing using least squares regression method and statistical analyses to validate the results were performed using ANOVA. The repeatability (intra-day) was evaluated comparing results of three eight-plate assays, performed in the same day at the same conditions, and the

intermediate precision was evaluated comparing results of three different days of experiment, being three assays of eight plates a day. Relative standard deviation (RSD) was calculated.

2.2.5.2. Accuracy

The accuracy was assayed by recovery method. Known quantities of standard solutions were added to sample solutions with nominal concentrations of 0.5; 1 e 2 µg/mL. The amount of standard added corresponds to 10% of these solutions concentrations. Then, the recovery percentages of the standard added were calculated. The results from three eight-plate assays performed in the same day were used in the calculations. Relative standard deviation (RSD) from the analyses was also calculated.

3. Results and Discussion

3.1. Optimization of the methodology

The success in a method validation is widely dependent of well-established analytical conditions. In particular case of microbiological methodologies, where there is a great source of variations that may interfere on analysis, is important the optimization of the conditions in order to obtain a reliable and reproductive method. Development and validation of analytical methods has been a lot explored in quality control, because of their importance in pharmaceutical analysis [27]. It's increasingly important to have alternative methods which can be applied to several pharmaceutical dosage forms considering each drug-chemical characteristics, and suitable to the different routines of the pharmaceutical laboratories [29]. The microbiological assay using the cylinder-plate assay and 3x3 design (three concentrations of standard and sample in the same plate) has been recommended by official references [17], being used for many antibiotics of several groups. The principle of this method is the drug diffusion on an agar surface inoculated with a microorganism, producing a zone of inhibition

of growth that is measured and your size is a function of the drug concentration [21,22,30], that way, this assay also allows to determine the drug potency and detects small variances of their biological activity. Agar diffusion methods has been the methodology chosen for water-soluble antibiotics [28]. All the culture medium used for this study were selected based on similar methodologies described for others similar carbapenems: meropenem and doripenem [14,19].

The antimicrobial activity of Imipenem against Gram positive bacteria is demonstrated [1,22]. So, it was chosen as test microorganism one Gram positive bacteria widely distributed on hospital environment: *Staphylococcus epidermidis*. In last decades due to increase of immunocompromised population, bacteria as *Staphylococcus epidermidis* has won importance as common cause of many nosocomial infections [23]. Beside this, your availability on our lab as well its adequate growth in the culture media selected and reproducibility on measurement of halos also were considered. So, two different concentrations for the inoculum suspension in a preliminary experiment with 20 plates were tested, with 10 plates for each inoculum concentration: 1% and 2%. It was observed that 2% concentration achieved a better growth in conditions and media tested, getting a more uniform growth.

From this result, another preliminary experiment tested different dilutions for standard and sample using plates with 2% of inoculum concentration. For convenience, these concentrations will be referenced here in two groups, range 1 for concentrations 1.0; 2.0 and 4.0 µg/mL, and range 2 for concentrations 2.0; 4.0 and 8.0 µg/mL. It is very important for a better and more reliable measure of inhibition halos to have adequate separation between them in the surface layer. For it, it is important to found a range with a good response from geometric progression used in the calculations. The two ranges tested did not provide a good response in terms of reproducibility and inhibition halo sizes, as expected. In this case, it was observed a very large halo sizes. Based on these first preliminary results, we tested another experimental variation focused on concentration range, testing 0.5; 1 and 2 µg/mL. Now, the result was

satisfactory because allowed adequate separation and reproducible measure of inhibition halos, as illustrated (Figure 2). So, one additional experiment was performed with the defined analytical conditions to test the reproducibility, showing satisfactory results.

3.2. Method validation

The validation parameters were linearity, precision (repeatability and intermediate precision) and accuracy of the method. All analytical procedures followed what is described in official guideline from ICH (International conference on harmonization) [24] and current edition of Brazilian pharmacopoeia [18].

From linearity, the method aims to obtain a linear relation between the variables drug concentration and the drug response represented by inhibition halo sizes, for a determinate range of concentration [25]. For it, it is constructed standard curves that correlates inhibition halo sizes with drug concentration in logarithmic values. In this work it was constructed nine standard curves in three days of analysis, being three curves a day at the same conditions. The standard curves considered each one 8 plates, totalizing 24 plates at the same day. The range of drug concentration assayed was 0.5; 1.0 and 2.0 µg/mL. The means of halo diameters for each drug concentration were 15.20 mm (RSD = 2.87); 19.45 mm (RSD = 1.42) and 23.12 mm (RSD = 2.76), respectively. In agreement to official guidelines the *r* value (correlation coefficient) of the standard curves needs to be close or equal 1.0, in order to demonstrate the best linear relation between the variables. In this work all curves obtained a *r* value > 0.99, being considered very satisfactory. A standard curve obtained in third day of analysis with r = 0.9991 is illustrated (Figure 3).

The linear regression analysis performed by ANOVA (analysis of variance) was used to prove the linearity performance, and did not shown deviations from parallelism or significant differences from the medium values (p value < 0.05), as recommended in official guidelines

[18, 19]. The Table 1 illustrates the ANOVA applied to results of third day of analysis, considering 24 plates results (equivalent to three assays with 8 plates).

According to official codes, the evaluation of precision can be expressed in two levels: repeatability and intermediate precision. Repeatability demonstrates the variation between sequential determinations of the drug sample in assays performed in the same day under the same conditions while the intermediate precision shows the variation between determinations performed in at least two different days in the same laboratory, under the same analytical conditions, being expressed as RSD [25]. As preconized in 3 x 3 design of cylinder plate assay, during precision both commercial sample and reference standard were analyzed in all experiments and then, the imipenem content was calculated. To access repeatability and precision intermediate, results of six assays with 8 plates were considered in three days of analysis (three assays with 8 plates a day) and the calculated RSD were satisfactory in all the cases (RSD = 0.28-0.64). The mean drug content was 101.05% (RSD=2.49) (Table 2). As described in imipenem monograph found in US pharmacopeia, the powder for injection has to contain 90-115% of Imipenem and Cilastatin [17], being that way, all the results were considered satisfactory. Microbiological assay is classified as biological assay and generally, have a highly variation of results [26] in comparison with analytical methods like HPLC. Despite of this, the RSD values obtained in the purposed method were adequate, being below to 5% for all experiments, showing that this microbiological assay is suitable to determine imipenem in drug formulation despite the inherent variation of biological assays.

The accuracy of the method consists in the capacity to obtain results close to the real values, in other words, is the grade of agreement between experimental results and the results accepted [25]. In this proposed method, accuracy was demonstrated by recovery test as it is also described for other methodologies applied to other carbapenems and validated already [14,20]. The percentage of recovery obtained was between 101.70 – 105.20% (mean recovery =

104,86%) with RSD= 2.95 (Table 3), each result considering three assays with six plates each one. So the recovery percentage obtained was considered satisfactory to determine imipenem.

Another validation parameter could be studied is robustness, which it is very worked in physicochemical methods and express the capacity of the method to resist to small variations in analytical conditions [25,27]. However, considering a bioassay whose results are dependent of microorganism and intrinsic variations are common in analytical routine, this parameter is not mandatory. Despite of the microbiological assays have a greater variation, these variations cannot compromise the analytical determination of the drug, regarding to their validation parameters, therefore it is essential to keep the behavior and execution of the method under very attention [20]. Considering that microbiological assays have variations presents during all process, and that in most cases, the authors referenced in this work did not describe robustness determination for the same methodology [14,20] we decided, in the first moment, do not include the study of this parameter on this validation.

4. Conclusions

This paper proposed the development and validation of a microbiological assay able to determine imipenem in powder for injection, having a good analytical performance from execution of the validation protocol. From all results already presented, the method proposed was considered validated because it demonstrated linearity, precision and accuracy. So, the microbiological cylinder-plate assay can be utilized in quality control routine applied to quantitation of imipenem in powder for injection, being an alternative for usual analytical methods like HPLC.

Conflict of Interest

The authors declare the absent of any conflict of interest in all the results presented in this paper.

Acknowledgements

The authors thank to Universidade Federal do Rio Grande do Sul and the Laboratório de Controle de Qualidade Farmacêutico for all structure that allowed to realize this work.

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SUPPLEMENTARY MATERIAL

Figure legends

Figure 1. Chemical structure of Imipenem.

Figure 2. Illustrative plate with 2% of concentration of inoculum obtained from three concentrations of imipenem standard (P) and sample (A): A1 and P1 for 0.5 µg/mL; A2 and P2 for 1.0 µg/mL and finally, A3 and P3 for 2.0 µg/mL. Growth on antibiotic media number 11, obtained during development of a quantitative microbiological assay for imipenem in powder for injection./

Figure 3. Standard curve correlating diameter of inhibition zone (mm) versus logarithmic of drug concentration (µg/mL), obtained from microbiological assay developed to quantitation of imipenem in powder for injection.

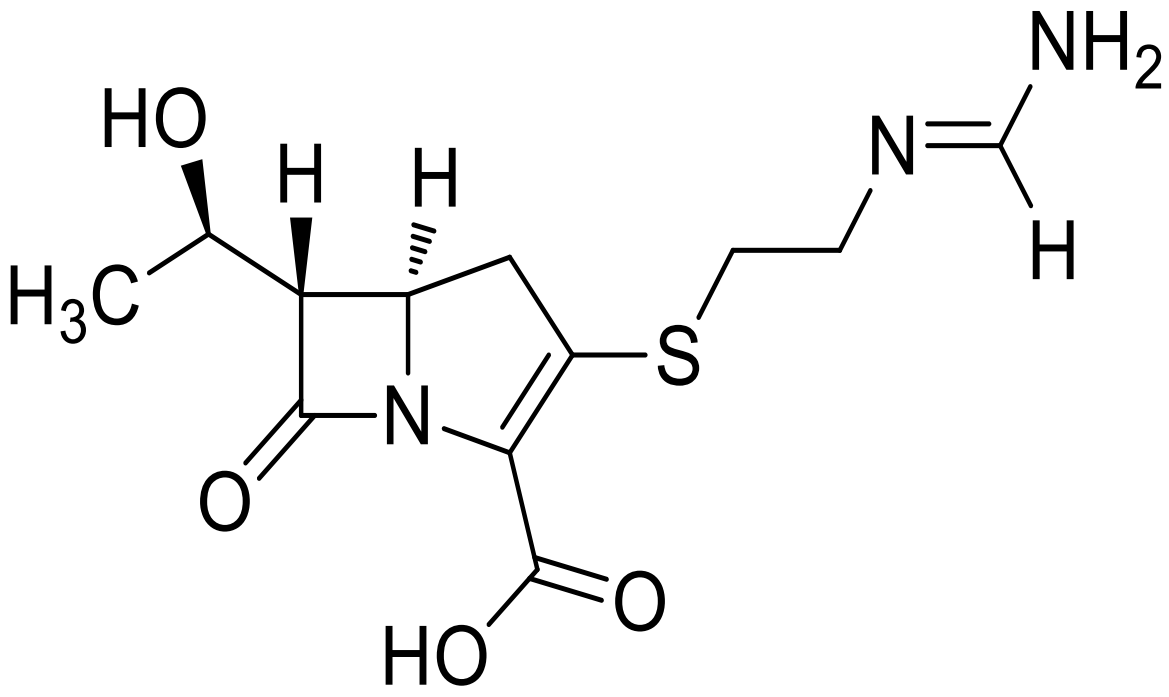

Figure 1

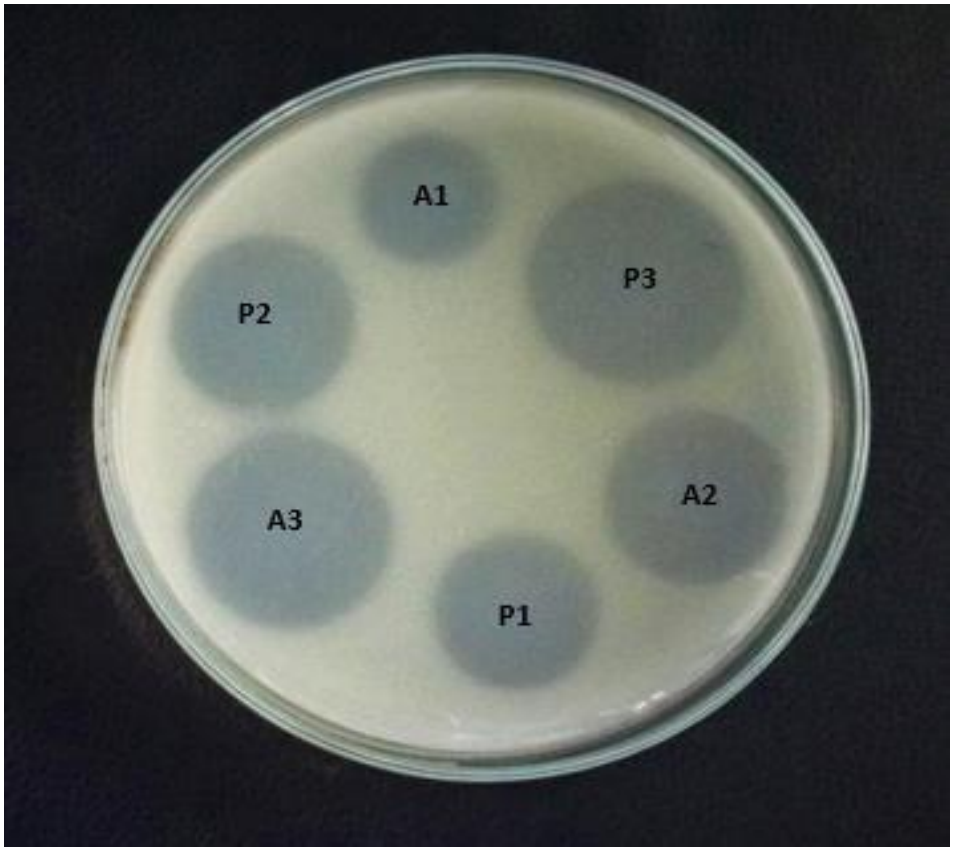

Figure 2

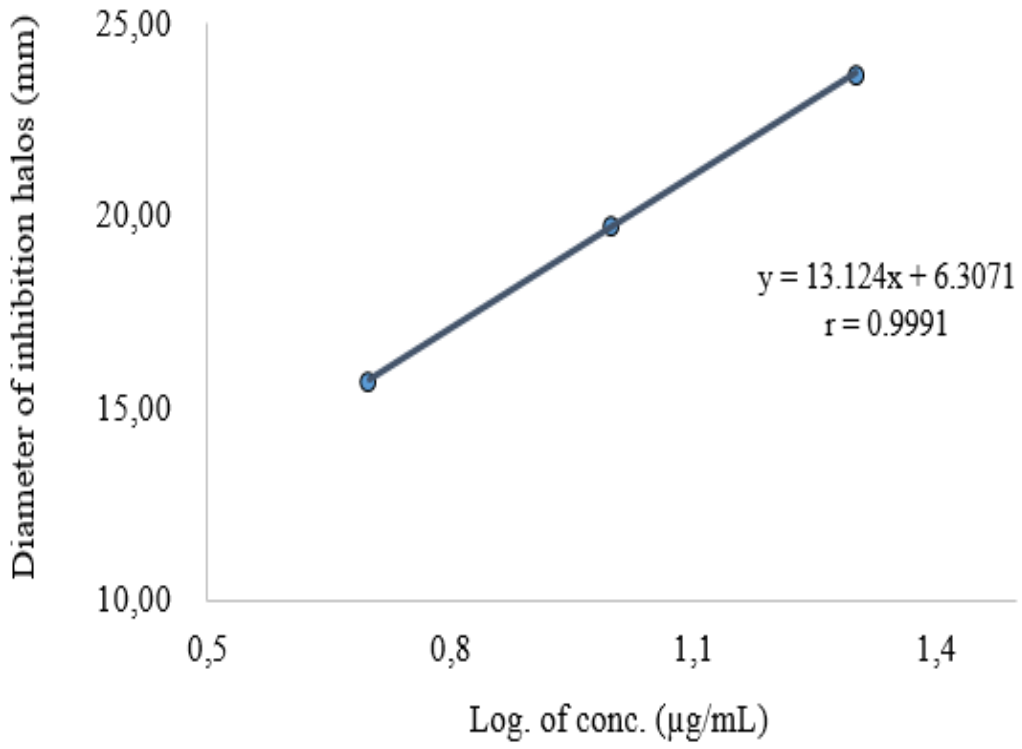
Diameter of inhibition halos (mm)
25,00
20,00
15,00
10,00
0,5
0,8
1,1
1,4
Log. of conc. (µg/mL)
y = 13.124x + 6.3071
r = 0.9991

Figure 3

Table 1. Analysis of variance (ANOVA) from quantitative results of linearity (third day) obtained during validation of microbiological assay for imipenem in powder for injection.

Variation sources	Degrees of freedom	Sums of Squares	Mean Squares	F-calculated	F-value
Preparation	1	0.005	0.005	3.88	4.12
Regression	1	509.60	509.60	395424.10	4.12
Desviation from paralelism	1	0.000	0.000	0.010	4.12
Quadratic	1	0.003	0.003	2.53	4.12
Quadratic difference	1	0.000	0.000	0.013	4.12
Difference between doses	5	509.61	101.92	79086.11	2.48
Difference between plates	7	0.009	0.001	0.951	2.29

Table 2. Results obtained from precision intermediate (inter-day) and repeatability (intra-day), studied during validation of microbiological assay for quantitation of imipenem in drug formulation. Data expressed as potency (%) and RSD.

	Repeatability		
	Antimicrobial[a] Potency (%)	Mean Potency (%)	RSD (%)
Day 1	104.16 104.16 104.67	104.33	0.28
Day 2	98.59 99.81 99.96	99.45	0.76
Day 3	98.98 98.99 100.09	99.35	0.64
	Intermediate precision	101.05	2.49

[a] Each result is a mean from three assays of eight plates.

Table 3. Mean values (%) of recovery test obtained from microbiological assay applied to quantitation of imipenem in powder for injection.

Sample concentration (µg/mL)	Standard added (µg/mL)	Recovered Concentration (µg/mL)	Mean of recovery [a] (%)
0.5	0.05	0.051	101.70
1.0	0.10	0.107	107.90
2.0	0.20	0.210	105.20
	RSD (%)		2.95

[a] Each result is a mean from three assays of six plates.

ANEXOS

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Acknowledgements

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[1] Luthy, I.A.; Bruzzone, A.; Pinero, C.P.; Castillo, L.F.; Vazquez, S.M.; Sarappa, M.G. Adrenoreceptors: non-conventional target for breast cancer? *Curr. Med. Chem*., **2009,** 16(15), 1850-1862.

[2] Geidl, W.; Pfeifer, K. Physical activity and exercise for rehabilitation of type 2 diabetes. *Rehabilitation,* **2011**, 50, 255-265.

Book Reference

[3] Crabtree, R.H. The Organometallic Chemistry of the Transition Metals. 3rd ed. New York: Wiley & Sons **2001**.

Book Chapter Reference

[4] Wheeler, D.M.S.; Wheeler, M.M. Stereoselective Syntheses of Doxorubicin and Related Compounds In: Studies in Natural Products Chemistry; Atta-ur-Rahman, Ed.; Elsevier Science B. V: Amsterdam, **1994;** Vol. 14, pp. 3-46

Conference Proceedings

[5] Jakeman, D.L.; Withers, S.G.E. In: Carbohydrate Bioengineering: Interdisciplinary Approaches, Proceedings of the 4th Carbohydrate Bioengineering Meeting, Stockholm, Sweden, June 10-13, 2001; Teeri, T.T.; Svensson, B.; Gilbert, H.J.; Feizi, T., Eds.; Royal Society of Chemistry: Cambridge, UK, **2002**; pp. 3-8.

URL(WebPage)

[6] National Library of Medicine. Specialized Information Services: Toxicology and Environmental Health.http://sis.nlm.nih.gov/Tox/ToxMain.html (Accessed May 23, **2004**)

Patent

[7] Hoch, J.A.; Huang, S. Screening methods for the identification of novel antibiotics. U.S. Patent 6,043,045, March 28**, 2000**.

Thesis

[8] Mackel H. Capturing the spectra of silicon solar cells. PhD Thesis, The Australian National University, Canberra, Australia, December **2004**.

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