

Mupirocin ointments: *In vitro* x *In vivo* bioequivalence evaluation

Stephanye Carolyne Christino Chagas¹, Camila de Almeida Perez Pimenta¹,
Juliana Kishishita¹, Irla Carla França Barbosa¹, Danilo Cesar Galindo Bedor¹,
Katia Aparecida da Silva Aquino², Davi Pereira de Santana¹, Leila Bastos Leal^{1*}

¹Department of Pharmaceutical Science, Federal University of Pernambuco, Recife, PE, Brazil

²Department of Nuclear Energy, Federal University of Pernambuco, Recife, PE, Brazil

Bioequivalence (BE) assessment of topical drug products is a long-standing challenge. Agencies such as the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have published several drafts in recent years suggesting different approaches as alternative to evaluate the BE. A proposed Topical Classification System (TCS) has even been discussed. Given the above, the objective of this research was to use *in vitro* and *in vivo* BE approaches to evaluate Brazilian marketed mupirocin (MPC) ointments, previously classified as TCS class 1. The *in vitro* permeation test (IVPT) was performed by applying formulations to pig skin by Franz cells. The *in vivo* methodology was dermatopharmacokinetic (DPK). These approaches (*in vivo* tape stripping and IVPT) demonstrated capability of distinguishing among different formulations, thus making them useful methodologies for BE evaluation.

Keywords: Mupirocin. Topical bioequivalence. Tape-stripping. Skin. Stratum corneum sampling *in vivo*.

INTRODUCTION

The Bioequivalence (BE) approach to evaluation of topical drug products has been difficult and challenging. Requirements for registration are different from country to country. (Kanfer, 2010; Braddy *et al.*, 2015; Soares *et al.*, 2015). In Brazil, the requirements for registration of a generic product are pharmaceutical equivalence to the reference product and that the composition of the generic formulation must contain excipients with the same composition and functions as those in the reference product (Anvisa, 2011).

In the United States of America (USA), for example, for topical drug products for local action, except for topical corticosteroids, the only recognized method to establish BE is by clinical studies, which are time-consuming and expensive (Jain, 2014; Yacobi *et al.*, 2014; Krishnaiah *et al.*,

2014; Raney *et al.*, 2015). In 1998, the United States Food and Drug Administration (FDA) published a draft guideline proposing a dermatopharmacokinetic (DPK) approach, in which drug levels in the stratum corneum (SC) are measured as a function of time post-application and post-removal of the product using tape-strip sampling *in vivo* in humans (FDA, 1998). In May 2002, the FDA withdrew this document, partly because a comparison study of a reference tretinoin gel with a bio-inequivalent product produced contradictory results (Pershing, 2001; Conner, 2001; Franz, 2001; FDA, 2002). So, the FDA initiated studies to identify sources of variability in the method, and to optimize the test procedure in order to minimize the number and complexity of the required tests while producing the required information for making a regulatory decision (Herkenne, 2008; N'Dri-Stempfer *et al.*, 2009). In an attempt to change this situation, the FDA has published several drafts in recent years suggesting alternative ways to evaluate the BE of products containing different drugs, and from different pharmaceutical forms including ointment (FDA 2002; FDA, 2012; FDA, 2016a; FDA, 2016b; FDA, 2017a; FDA, 2017b).

*Correspondence: Departamento de Ciências Farmacêuticas (NUDFAC). Universidade Federal de Pernambuco. Rua Prof. Arthur de Sá, S/N. Cidade Universitária, Recife. CEP.: 51011-000, PE, Brasil. Phone: +44.33026590. Email: leila.leal@nudfac.com.br. ORCID:<http://orcid.org/0000-0002-1252-8000>

According to the draft for acyclovir ointment (published in 2012), for example, the BE between the reference and the tested formulation could be established based on the similarity of Q1 (similar qualitative composition of excipients), Q2 (similar quantitative composition), and Q3 (similar microstructural arrangement) (FDA, 2012). Thus, according to the proposed topical TSC, the formulations can be classified: class 1 (Q1, Q2 same and Q3 same), class 2 (Q1, Q2 same and Q3 different), class 3 (Q1, Q2 different, and Q3 same) and class 4 (Q1, Q2 different, and Q3 different). On the other hand, the European Medicines Agency (EMA) recently published a draft guideline on quality and equivalence of topical products, with the consultation period ending on 30 June/2019. This publication considers *in vitro* human skin permeation (IVPT), *in vivo* stratum corneum sampling (by the tape stripping approach) and *in vivo* vasoconstriction assay for corticosteroids (EMA, 2019).

Considering the above mentioned, the aim of this present research was to apply different approaches, regardless the national or international draft guidelines or regulatory guidelines, to evaluate Brazilian marketed mupirocin (MPC) ointments (reference, generic A, generic B and similar), previously classified as Class 1 (Q1, Q2 and Q3 similar) (Cavalcanti *et al.*, 2019).

MATERIAL AND METHODS

Chemicals and reagents

MPC was kindly donated by Cristália Produtos Químicos Farmacêuticos LTDA (purity 99.6%) and used as the reference chemical. Hydrochlorothiazide obtained from Instituto Nacional de Controle de Qualidade em Saúde (INCQS, Rio de Janeiro, Brazil) was used as an internal standard (IS). All the solvents and reagents used in the analyses were HPLC grade.

Formulations and Topical Drug Classification System

Mupirocin ointments 1% were purchased on the Brazilian market (Bactroban®, Generic A, Generic B

and Similar). Their composition and physical-chemical characteristics were described by Cavalcanti *et al.*, 2019. According to Cavalcanti *et al.*, the formulations were classified as TCS class 1, however the similar product did not present the requirements for similarity (FDA, 1997).

Fourier-transform infrared spectroscopy (FTIR)

Infrared spectra of the samples were obtained by Fourier Transform Infrared Spectroscopy using a Jasco FTIR – 4600 Fourier Transform Infrared Spectrometer (Tokyo, Japan), equipped with an attenuated total reflection accessory (FTIR-ATR), operating at a resolution of 4 cm⁻¹, ranging from 400 to 4000 cm⁻¹, and at a rate of 32 scans/spectrum. FTIR-ATR spectra were used to analyze the marketed drug products.

LC-MS/MS method

The mass spectrometer Sciex 3200 QTRAP (SCIEX, Toronto, Canada) infusion experiments were performed for multiple reaction monitoring (MRM) optimizations with a Harvard Apparatus 11 elite syringe pump (Holliston, MA) at a flow rate of 10 µL.min⁻¹. An MRM method was prepared including the most intense transitions for MPC and the IS.

The mass spectrometric parameters were optimized to obtain maximum sensitivity for unit resolution. The electrospray ionization (ESI) was performed in negative mode. The following transitions were monitored in MRM mode: m/z 499.3 > 173.2 for MPC and m/z 295.8 > 204.9 for IS.

Quantitation experiments were performed using a 20A LC System (Shimadzu Corporation, Kyoto, Japan) equipped with two analytical pumps (LC-20AD), a vacuum degasser (DGU-20A3), an autosampler (SIL-20AC HT), and a controller module (CBM 20A). The chromatographic column used was a SunFire C18, 50 x 2.1 mm, 3.5µm (Waters®, Massachusetts, USA) kept at 40 °C. Isocratic elution was achieved using a mobile phase consisting of water/methanol (15:85 v/v) operating at 0.4 mL.min⁻¹. The retention time was 0.7 min for MPC and 0.4 min for IS with a total run time of 2 min.

The calibration curve was prepared in methanol solution in a linear range with six concentration points: 10.0; 25.0; 50.0; 100.0; 200.0 and 300 ng. mL⁻¹. The quality controls were prepared as calibration curve at 30.0 (low quality control - LQC); 150.0 (medium quality control - MQC) and 240.0 (high quality control – HQC). System control and data acquisition were performed with Analyst® 1.5.2 software including the “Explore” option (for chromatographic and spectral interpretation). Calibration curves were constructed with the MultiQuant software version 2.1. using a linear weighted least squares (WLS) regression using $1/x^x$.

Validation was carried out according to the Brazilian Health Surveillance Agency (ANVISA), guidelines for bioanalytical assays (Anvisa, 2012).

***In vitro* skin permeation test (IVPT)**

A single dose (100 mg) of each ointment was applied to dorsal pig skin (1.77 cm² area exposed) mounted on a Franz cell (Vision® Microette). The skin was sourced from a local abattoir at Paulista-Brazil, dermatomed (Zimmer dermatome, Dover, DE) to a thickness of approximately 750 µm and then frozen at -20°C. The donor compartments were kept occluded and the receptor

solution (of about 6mL) consisted of phosphate buffer (25 mM) pH 7.4, which was kept under constant stirring by a magnetic bar. Fourteen replicates were evaluated for each formulation and after 6 hours study, the residual formulation was removed from the skin by a cleaning procedure using swabs (Biosoma®) containing isopropyl alcohol, twice. Then, after progressive removal of SC by the tape stripping method (20 tapes were used for each site), the MPC was extracted from the tapes. At the end of procedure, MPC was extracted from each section of pig skin (viable epidermis and dermis), to evaluate drug retention and the receptor liquid was collected for quantification.

Bioequivalence evaluation by SC tape-stripping experiments

The study protocol (CAAE 34657814.2.0000.5208) was approved by the local ethics committee of the Federal University of Pernambuco, Recife, Brazil. Fourteen healthy volunteers (11 women and 3 men) were informed about the procedure and signed a consent term to participate of the study (Cordery *et al.*, 2017; Araújo *et al.*, 2018). Their mean age, weight, and height were 25 ± 5 years; 66.5 ± 17.2 kg; and 164 ± 6 cm, respectively.

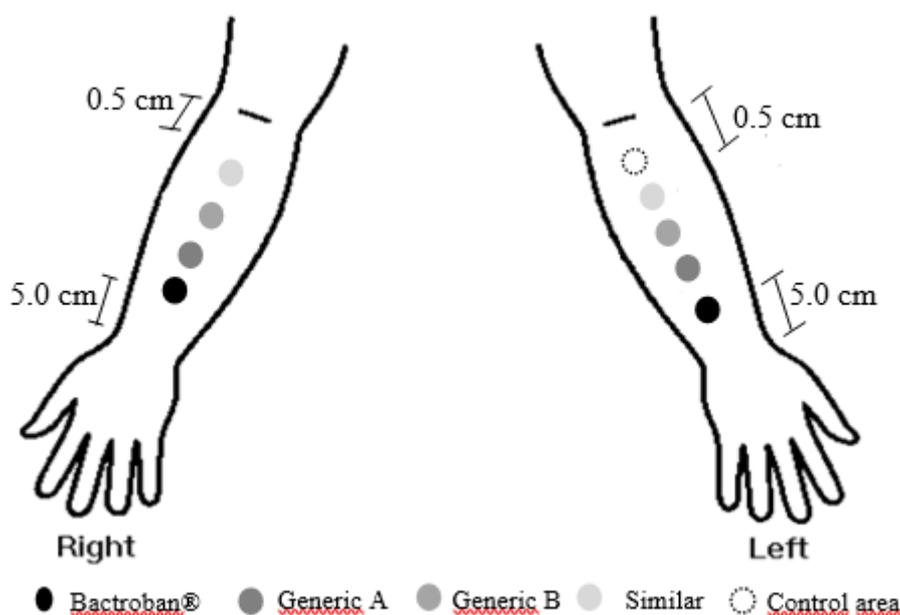


FIGURE 1 - Application sites of the formulations marked on the ventral portion of each.

The tape stripping protocol was used to evaluate MPC in a single application (6hs dose duration) and was adapted from N'Dri-Stempfer and collaborators (2008) (N'Dri-Stempfer, 2008). The test was performed on the forearm between at least 5 cm above the wrist and a minimum of 0.5 cm below the bend of the elbow. One hour before starting the study, the forearms of each volunteer were cleaned and dried. A dose of 25 mg / cm² (reflecting the recommended use level) of each formulation was applied (Cordery *et al.*, 2017). Formulations were applied in duplicate, resulting in 9 skin sites per subject (two per formulation and one control) (Figure 1). Each application site was demarcated with a circular acetate template with an area of 2.54cm²; the tape stripping area was 1.77 cm². Transepidermal water loss (TEWL) was measured using a Tewameter (Courage + Khazaka electronic GmbH -CK electronic) at all sites to monitor the skin barrier. After 6 hours, the remaining formulation was removed from all application sites with two swabs containing isopropyl alcohol (Biosoma®) and the SC was progressively removed by tapes (Scotch Reserve tape, 3M, St. Paul, MN, USA) (up to 20 tapes were used for each site). The mupirocin present on each tape was subsequently extracted and analyzed by the analytical method described below.

Drug extraction procedure

The tapes were organized in groups (1-2; 3-5; 6-8; 9-12; 13-16; 17-20) of tape (Scotch Book Tape, 3M Co., St. Paul, MN, USA) of both *ex vivo* (pig SC) and *in vivo* (human SC). After that, the tapes were spiked with 50µl of an IS solution and left to air dry, then it was added of 1mL of methanol in a controlled temperature bath (32°C) under constant stirring for 6 hours. After extraction, all samples were filtered (0.45µm HV-PVDF Millex©, Carrigtwohill, Ireland) and analyzed by the LC-MS/MS method (Cordery *et al.*, 2017; Araújo *et al.*, 2018).

For the retention study, each slice of pig skin (viable epidermis and dermis) used for the IVPT were cut into smaller sections and the same extraction procedure was used as for the tapes.

Interpretation of results

The statistical analysis for IVPT involved two-tailed Student's t-tests and one- and two-way analyses of variance (ANOVA) followed by Bonferroni's test; p-values less than 0.05 were considered statistically significant.

For the *in vivo* study, the bioequivalence analysis was performed following the published approach of N'Dri-Stempfer *et al.* (2008) (N'Dri-Stempfer *et al.*, 2008). Briefly, the average (for log transformed data) of the duplicate drug mass measurements for each product was calculated in each subject, and then the geometric mean (the anti-log of the average of the log transformed values) and the lower and upper 90% confidence interval of the geometric mean were calculated. The tested formulations (Generic A, B and similar) were considered bioequivalent to the referenced Bactroban® ointment when the ratio (\pm the 90% confidence interval) of the amount of drug in the SC from the test product to that from the reference formulation was within the range of 0.8–1.25.

RESULTS AND DISCUSSION

Liquid chromatography tandem mass spectrometry (LC-MS/MS) method

The applied method demonstrated excellent specificity with no endogenous interferences at the retention times for MPC and the IS, even with a low chromatographic retention and an analytical run time of 2 min.

The cross-talk test showed no interference between the MRM channels of MPC and the IS despite the low chromatographic resolution between the compounds analyzed. The carry-over test showed no interference between the samples in an analysis sequence.

For the linearity evaluation, weighted least square linear regression was used to obtain the linearity over one order of magnitude (10–300 ng/mL (1/x^x)), with a mean determination coefficient of 0.9994 ± 0.0003 (n=6).

Intra- and inter-day precision and accuracy obtained from human and pig skin tapes (Table I) were within the acceptable limits set by the guidelines for validation of bioanalytical methods.

TABLE I - Accuracy results for human and pig tape

Concentration (ng/mL)	Precision and accuracy results of validation method					
	Intra-day ($\mu\text{g/mL}$) (n=5)		Inter-day ($\mu\text{g/mL}$) (n=15)		Inter-day ($\mu\text{g/mL}$) (n=15)	
	Human	Pig	Human		Pig	
	Mean (R.S.D.)	Mean (R.S.D.)	Precision R.S.D.(%)	Accuracy R.E. (%)	Precision R.S.D.(%)	Accuracy R.E. (%)
10	11.92 \pm 0.54	11.3 \pm 1.86	4.53	19.20	16.49	13.00
30	34.18 \pm 2.15	33.8 \pm 2.52	6.28	13.93	7.46	12.67
150	153.33 \pm 13.88	165.40 \pm 8.73	9.05	2.22	5.28	10.27
240	232.4 \pm 8.87	253 \pm 23.02	3.82	3.17	9.10	5.42

TABLE II - Mean values for recovery of the stratum corneum (spiked samples) and pig skin 450 (epidermis and dermis).

Concentration (ng/mL)	Stratum corneum			Epidermis and dermis		
	Mean \pm SD (ng/mL)	CV (%)	Recovery (%)	Mean \pm SD (ng/mL)	CV (%)	Recovery (%)
30	29.83 \pm 0.61	2.05	99.44	29.27 \pm 1.01	3.44	97.56
150	149.00 \pm 1.00	0.67	99.33	149.57 \pm 1.25	0.84	99.71
240	238.67 \pm 7.51	3.14	99.44	230.00 \pm 8.00	3.48	95.83

According to the recovery evaluation and the post-extraction spiked samples, the results of the matrix effect tests showed no significant influence of the biological matrix on the MPC and IS analytical response. Therefore, ion suppression or enhancement by the SC was negligible for this method.

The data obtained for MPC are in accordance with the acceptable limits set forth in the guideline for bioanalytical methods of validation (ANVISA,2012).

FTIR

Many studies have discussed the different ways to evaluate Q3 (Kryscio *et al.*,2008, Shah *et al.*, 2015, Ehtier *et al.*, 2018). The data related to MPC formulation, such as viscosity, spreadability, and IVRT demonstrated that although the two first tests show statistically significant differences ($p < 0.05$) for all products, their calculated

release rate was not affected when the release results were calculated according to the SUPAC SS 1724 guide (FDA, 1997). There was an exception for the similar formulation. The British Pharmacopeia, 2008, describes this as a product in which the drug content is below the established limit. The probable explanation is the manner in which the drug is bound into the microstructure of the semi-solid formulation, an essential quality.

The FTIR-ATR spectra of MPC ointment (reference, generic A and generic B and similar) in the 4000 - 500 cm^{-1} region performed in this research are shown in Figure 2. These were compared to the spectra of polyethylene glycol (PEG) and pure MPC. PEG is commercially available over a wide range of molecular weights from 300 g/mol to 10.000,00 g/mol (French, Thompson, Davis, 2009). In this study the ointments were produced with PEG in the range from 400 g/mol (PEG 400) to 4.000 g/mol (PEG 4000).

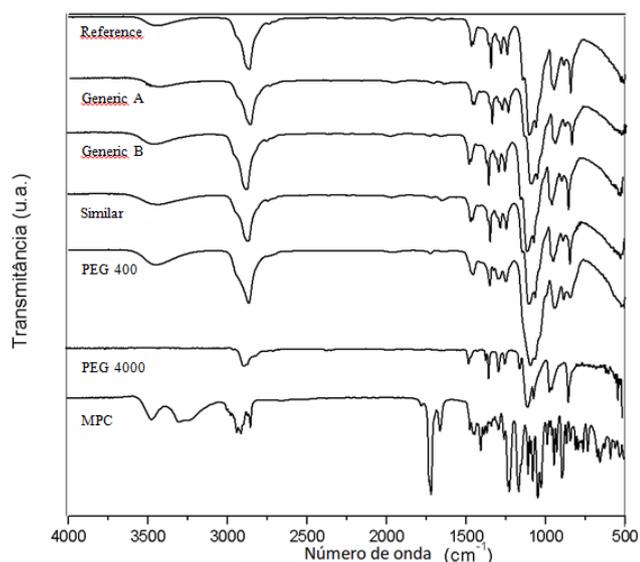


FIGURE 2 - FTIR-ATR spectra of MPC ointments, PEG 400, PEG 4000, and MPC drug.

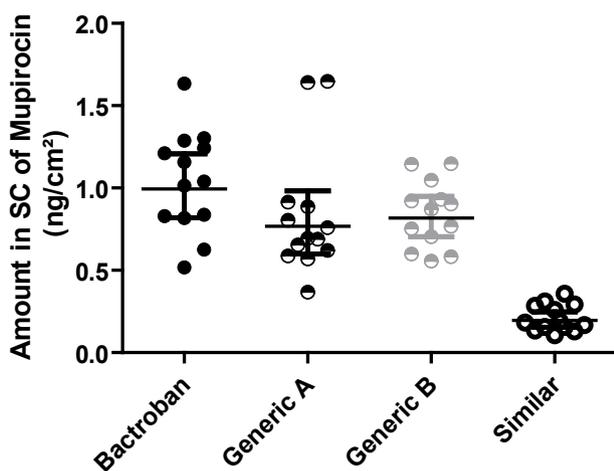
Principal peaks found in the spectra of MPC ointments, PEG, and pure MPC were 3400-3437 cm^{-1} attributed to O-H group, 2900-2940 cm^{-1} attributed to C-H stretch, 1460 cm^{-1} attributed to $-\text{CH}_2$ scissor, 1300-

1200 cm^{-1} attributed to C-O stretch and 1100-1060 cm^{-1} attributed to C-O-C stretch. The peak at 1750 cm^{-1} attributed to C=O carbonyl group was found only in the MPC spectrum.

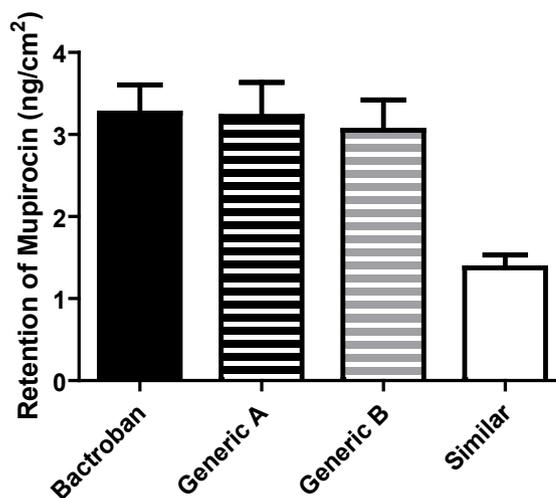
As expected, no differences were found between PEG 400 and PEG 4000 spectra. On the other hand, the spectra of MPC ointment systems were very similar to spectral patterns of PEG, i.e., there were no appearance peaks attributed to MPC drug in MPC ointment spectra. This result means no interactions occurred between the MPC drug and PEG, probably due to low amounts of MPC in the ointments, regardless of any other aspects noted above (viscosity, spreadability, etc.).

In vitro skin permeation test (IVPT)

The results of the IVPT experiments are summarized in Figure 3, which shows the MPC mass in SC and in underlying tissue of the 14 pieces of dorsal pig skin exposed to each MPC ointment. Regardless of the formulations used, the drug did not reach the receptor solution (10 ng/ml limit of quantification).



A



B

FIGURE 3 - Amount of MPC ($\text{ng}/\text{cm}^2 \pm \text{SD}$) in the stratum corneum (panel A) and epidermis and dermis (panel B) in dorsal pig skin ($n=14$), for the 4 formulations.

Porcine skin was used in this IVPT experiments due to its reliability as a model for the human barrier

(Sekkat, Guy, 2001), and because of the limited availability of human skin. Besides this, the IVPT has

not yet been accepted as test material by regulatory agencies as a surrogate for *in vivo* bioequivalence, among other uses, due to the lack of metabolic activity. In this study, drug retention (amount of MPC in the viable epidermis and dermis) and the amount of mupirocin in the SC showed no statistically significant difference among different formulations (Friedman's ANOVA and Bonferroni test) except for the similar products. For MPC in SC, the results were the same, whether or not the two first tapes were included. This study demonstrates the ability of this approach to demonstrate the similarities and differences between topical formulations. The same has been established for betamethasone valerate and econazole, butenafine hydrochloride, ketoconazole, clobetasol propionate topical formulations (De Freitas *et al.*, 2015; Mitra *et al.*, 2016; Leal *et al.*, 2017; Soares *et al.*, 2018).

Bioequivalence evaluation by SC tape-stripping experiments

Five volunteers presented skin phototype II and V and nine volunteers had skin phototype III and IV. At the end of the study, all the volunteers presented temporary redness in some places after the tape stripping procedure.

Following the same calculation performed by N'Dri-Stempffer and collaborators (2008) (N'Dri-Stempffer *et al.*, 2008), bioequivalence was evaluated after 6 hours of application of the reference product Bactroban® and the four products (generics A and B, and the similar) using the ratio of transformed log of drug amount (ng/cm²) (mean ± SD confidence interval 90%) (Table III). Figure 4 shows the intra-individual variability between duplicate applications, as well as inter-individual variability. As described by Cordery, 2017, "the variability observed between duplicate measurements should be due to differences between the skin sites, variability in application of the formulations, or variability in the efficiency of removing unabsorbed formulation at the end of the drug contact period" (Cordery *et al.*, 2017).

TABLE III - *In vivo* bioequivalence assessment ratio of products containing mupirocin (generic A, B and similar) compared to the reference, Bactroban®

Formulations	Generic A	Generic B	Similar
Mean	0.964	0.970	0.547
CI upper	1.053	1.173	0.782
CI lower	0.883	0.803	0.383

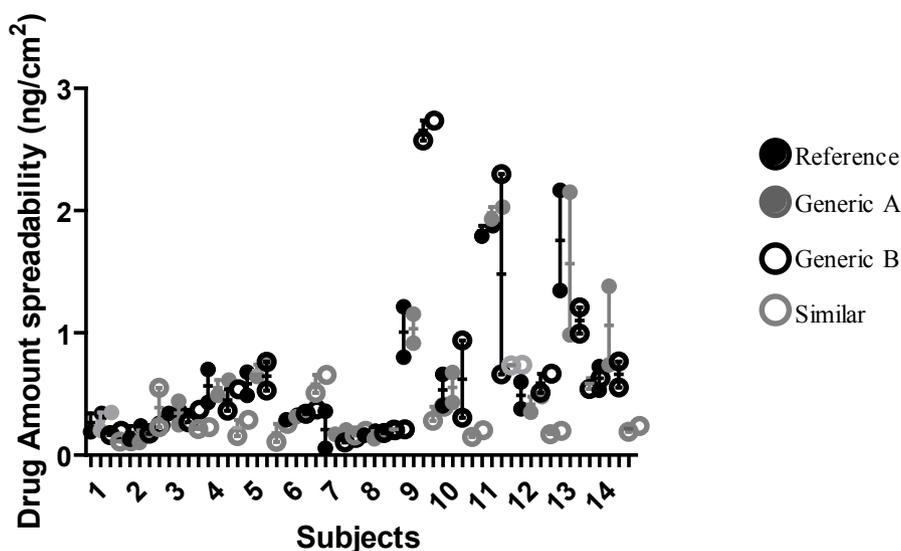


FIGURE 4 - Drug amounts per unit area ($\mu\text{g}/\text{cm}^2$) of the duplicate determinations in each subject for four MPC products measured after 6 hs contact.

In agreement with Araújo (2018), this bioequivalence protocol demonstrated that with only 14 subjects, was possible to evaluate the MPC bioequivalence (generating reproducible data) (Araújo *et al.*, 2018). Generics A and B can be considered bioequivalent to the reference product. The similar product (considered non bioequivalent) acted as a proof of the method concept, demonstrating the ability of this tape stripping approach to distinguish among different formulations, as was described by Nallagundla and collaborators in 2018 (Nallagundla, Patnala, Kanfer, 2018).

Several researchers have used the *in vivo* tape stripping technique, among them (Parfitt *et al.*, 2011; Leal *et al.*, 2017; Cordery *et al.*, 2017; Araujo *et al.*, 2018; Nallagundla, Patnala, Kanfer, 2018), aiming to demonstrate the ability of this method to assess bioequivalence between formulations containing drugs from different therapeutic classes.

The above mentioned-formulations used are qualitatively identical (Q1) and have also been shown to be similar in terms of microstructure (Q3), confirmed by IVRT rate (Cavalcanti *et al.*, 2019) and FTIR (Figure 1), being classified as a class 1 formulation (Cavalcanti *et al.*, 2019). Shah proposed in 2015 that these formulations should be waived from a clinical trial (Shah *et al.*, 2015). But, according to the mupirocin ointment FDA draft, there is only one recommended study - that is the Clinical Endpoint Bioequivalence (BE) Study (FDA, 2010). After performing the *in vivo* MPC tape stripping study, the bioequivalence could be evidenced and it would probably be the best decision to waive a clinical trial.

Therefore, considering the large number of methodologies (each one with its limitations), there is a growing idea about the use of a rational combination of techniques that can provide complementary evidence of the intended topical evaluation. According to Chang *et al.*, 2013, the choice of tests would depend on factors such as the complexity of the drug product (Chang *et al.*, 2013).

CONCLUSION

In this study, generic formulations A and B, were considered bioequivalent and the similar bio inequivalent, in both *in vitro* and *in vivo* outcomes. These simple approaches demonstrate methodological capacity to

indicate similarities and differences between formulations using only fourteen skin samples and fourteen volunteers, demonstrating the described method to be a potentially complementary technique to evaluate the bioequivalence of topical drug products.

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