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A Pharmaceutical Biotechnology Experience: Bacterial Nanocellulose for Transdermal Drug Delivery Systems Design. Safety and Organoleptic Acceptance Studies

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INTRODUCTION

Nanotechnology introduces sophistication to traditional pharmaceutical technology. A blank canvas that offers unexplored opportunities, where the most subtle physical-chemical interactions introduce significant changes in the behavior of the ingredients in a formula. An example is the plethora of possibilities offered by the incorporation of nanocellulose in bioinspired products design. Nanoscale cellulose can be obtained through a top-down approach, starting from plant raw materials. This approach involves a variety of drawbacks such as indiscriminate logging, monocultures, complex separation processes, high volume of strong chemical substances, environmental pollution, desertification among others. [1] This energetically expensive process can be directly replaced by a bottom-up approach. It is possible to obtain bacterial nanocellulose (BNC) by biosynthesis using specific bacterial strains.[2] Bacterial species such as *Pseudomonas fluorecens*, *Gluconacetobacter xylinus* and *Gluconacetobacter hansenni* produce cellulose as one of their metabolites. *Gluconacetobacter xylinus* is the chosen study model used to elucidate the biosynthetic mechanisms involved. [3] A film of cellulose nanofibrils of controllable thickness is produced at the air-culture medium interface. [4,5] Cellulose is a primary metabolite synthesized within of the bacterial cell. The chains are twisted into nanofibrils and mechanically amplified. [6,7] During biosynthesis, van der Waals forces and hydrogen bonds between the hydroxyl groups and oxygen of adjacent molecules promote the parallel stacking of multiple cellulose chains that form elemental clusters of fibrils to aggregate into larger microfibrils. [8] BNC has distinctive characteristics due mainly to its size and fibrillar arrangement that introduces modifications in its biological and physicochemical properties, such as biocompatibility and biodegradability. In addition, BNC fibers are lighter, have greater optical transparency and their surface is chemically adaptable, allowing the union of multiple functional groups and improving their mechanical properties.[9,10] This biotechnological process allows us to obtain a raw material with distinctive

characteristics that position it as an interesting object of study due to the variability of its applications[1]. The field of designing new drug carrier systems for pharma is one of the most sought after. Also there is renewed interest in transdermal drug delivery. In addition to the technological advantages, it presents environmental and economic advantages compared to the traditional process. The economy of resources is based on significant energy savings, using static crops, fewer man hours, no chemical substances such as strong acids or bases are used and the waste obtained can be reinserted into the production cycle. It is an ecologically friendly process and the raw material obtained is biodegradable. [11,12]

Pharmacopoeias around the world incorporated in their annals pharmaceutical forms intended for temporal control of release: prolonged release, delayed release and pulsatile release (Royal Spanish Pharmacopoeia 3rd edition 2005; European Pharmacopoeia 6th edition, 2008; USP 32, 2009). [13] To these are added site-specific release formulations and new rapid disintegration forms. Excipients are non active pharmaceutical ingredients that play a key role, since the control of release is regulated by the materials that make up the system. [14] Transdermal drug delivery systems (TDDS) are designed to achieve a prolonged therapeutic effect with a single dose, through the continuous release of the drug over a certain period of time. [15] The evolution of pharmaceutical technology in this sense led to the design of 3 generations of TDDS where the objective is to enhance skin penetration. The first chemical generation refers to increasing the effectiveness of the drug by increasing doses, the second generation encompasses physical permeation technologies and the third generation uses penetration enhancers. [15] Transdermal drug delivery is an innovative research area that is becoming prominent globally as a result of its unique merits over other routes. The therapeutic efficacy of a drug delivered in a TDDS is based on: its non-invasive nature, the increase in the bioavailability of the drug, the ease of application, the absence of gastrointestinal side effects, its accessibility to the patient, its painless nature, bypassing the hepatic portal system and drug metabolization reduces the risk of cirrosis by intoxication. [16-19] TDDS is proposed as a substitute for oral drug transport. The dermis, highly irrigated, is transformed into a mighty river with multiple tributaries composed of arterioles and venules that facilitates the constant passage of the drug into the systemic circulation and enhances its effects.

In this work we present the design of a nanoemulsion with filmogenic capacity based on BNC as a non-active pharmaceutical ingredient. The objective was to demonstrate the capacity of the designed formula to form filmogenic films useful as TDDS, its safety and its solubility in water. Self-application, controlled release capacity and acceptability of the formula by the patient were the selection criteria. For this work, a pure drug model was used, in order to facilitate monitoring of controlled release. Safety and acceptance studies of organoleptic characteristics were carried out in a panel of 50 healthy skin volunteers.

Key words: Bacterial nano cellulose, Control released formula, Topical formulation, Nanobiotecnology.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A Pseudomonas fluorescens WS strain (wrinkly spreader) (donated by PhD Andrew Spears, School of Science, Engineering and Technology, Abertay University) It was grown in King B broth (Britania-C.A.B.A-Argentina) and kept at 25ºC.

BNC production process

For BNC production, static cultivation was used. All growing test were made on petri dishes with 50 mL of fresh medium 72h at 28±1°C. All BNC obtained were washing with distillate water to remove medium. BNC was centrifuged 20 min at 8000rpm. Then 1mL of NaOH 2% v/v was added and autoclaved 15min at 121ºC, these procedure is made for detach some bacterial cell which could by immerse on BNC. BNC were washed with distilled water until neutralization. [20]

BNC dry weight

Dry weight was measured in dry films. The results were reported as 'production' and expressed in weight of dry BNC per liter of King B medium (g/L). [20]

Filmogenic emulsion material

Sephigel 305, metacrilate polymer (Fabriquimica-Buenos Aires, Argentina), cosgard type preservative, *Gentamicine sulfate*, (Eiffel Química-Buenos Aires Argentina) BNC, flax seed extract, glicerol.

Formulation design [21]

Formulation studies were carried out in order to evaluate pharmacotechnical aspects such as: pH, homogeinity, viscosity, organoleptic properties and stability were developed as required on Administración Nacional de Medicamentos, Alimentos y Tecnología Médica (ANMAT) disposition Nº7667.

Homogeneity assays

The filmogenic emulsion designed was applied as a thin layer on polypropylene slides. The homogeneity was tested by visual appearance after application. The same formulation (3mL) was used for centrifugation assays (15 min, 3500 rpm) and separation in phases was controled [22] Under these conditions the homogeneity of the TDDS was qualitatively qualified as follows: very good (no phase separation), good (appearance of small volume of supernatant), regular (phase separation withslight appearance of clotted) and poor (separation of the phases with appearance of pellet).

pH measurements

The pH of the filmogenic emulsion was measured in a pHmeter (Broadley James Corporation, Irvine, CA) by dipping the glass electrode into the TDDS. [21]

Rheological studies

The viscosity was determined and measured as centi Poise (cP) at 25 ºC (stored temperature) and 32ºC (healthy skin temperature) by using a Cannon viscometer with spindle Nº8 from 3 rpm to 60 rpm (Cannon Instrument Company, LV2000 model, Pennsylvania, EEUU). [21,22]

***In vitro* passive permeation studies** [23]

This assay was conducted using vertical type Franz diffusion cells having a receptor compartment capacity of 10 mL. Cellulose membranes (D9527 avg. flat width 43 mm (1.7 in.) Sigma Aldrich Chemical CO., St. Louis, MO) were mounted between the half-cells in contact with receptor fluid (0.9% NaCl) and were equilibrated for 1 h. The area available for diffusion was 1.8 cm^2. The fluid in the receptor compartment was maintained at 32 ± 0.5 ºC (skin temperature). Semi-solid formulation (0.5 g) was placed in the donor compartment. The entire assembly was kept on a magnetic stirrer (100 rpm) and at each time four cells were removed from the system, aliquots (2 mL) of the receptor phase at specific time intervals (0,1, 2, 3, 4, 5, 6, 7, 8, 9 and 10h). Gentamicine Sulfate shows an absorbance peak at 400 nm. The OD_{400} of the solution was measured in a UV-Vis spectrophotometer (Thermo Spectronic Genesys 10 UV-VIS Rochester, NY).

[24] Cumulative amounts of gentamicin sulfate that permeated the diffusion unit surface (µg/cm^2) were plotted against time (h). The results were expressed as mean ± standard deviation (SD) (n =16).

Gentamicine sulfate cuantification [24]

The quantification of gentamicin sulfate was done by means of a colorimetric reaction with ninhydrin, which, being a strong oxidant, is capable of causing oxidative decarboxylation and deamination of the structure of the Gentamicin sulfate. The method is based on the reaction of ninhydrin (hydrate of tricetohydrindene) with the primary and secondary amines at temperaturas elevated, forming ammonia and carbon dioxide due to reduction of the ninhydrin to hydrindantine. When the reaction occurs, a compound of blue-purple coloration, called Ruthermann purple. The chromophore for the case of Gentamicine Sulfate can be detected at 400 nm.

Informed consent form development [25]

Informed consent sheet was designed by the protocols aproved for the World Health Organization and the provisions of the national regulatory entity National Administration of Drugs, Food and Medical Technology (ANMAT) Nº6677/10. [26]

Safety and organoleptic caracteristics acceptance studies in healthy skin volunteers [27]

Since there is no foreseeable risk from the use of BNC, nor other ingredient on the TDDS designed is posible and representative to evaluate the safety of their ingredients simply evaluating the appearance of edema and/or erythema (exploring potencial allergies) in a clinical test. The corrosion test in a model of healthy skin was selected [28] as a validated methodology by ANMAT (Resolution Nº 288/90)[24] in Argentina. This trial involved 50 healthy volunteers. The skin surface chosen was that recommended by the World Health Organization.

Test Method [29]

The human single closed patch test under occlusion was adapted and performed. Because it is an nano emulsion that generates an occlusive film on the skin after its applied. The test materials were occluded on the upper outer arms in two sets. The first set was occluded 4 hours if no signs of irritation occurred the second set would be further occluded for a total of 24 hours. After the required periods of skin contact, the patches were washed and observed for any signs of skin irritation 1 hour after removal, then 24 and 48 hours afterwards.

For the safety studies, the following parameters were considered:

a) Absence of dermal reaction, nor edema, erythema or skin dryness

b) Weak reaction described as a slight presence of edema and/or erythema, skin dryness in the application area was considered.

c) Moderate reaction described as the presence of edema or erythema in the application area.

For the analysis of the results of the evaluation of organoleptic characteristics, the drying speed perceived by the healthy volunteer was considered as:

a) Fast drying speed time as less than 1 minutes

b) Medium drying speed time as 1 to 3 min

c) Slow drying speed time 3 minutes or more

Other organoleptic characteristics achieved were those whose perceptions increase the user's comfort such as a pleasant sensation, no pulling effect, presence of cracks or folds.

Ethical considerations

This study meets the ethical and scientists standards to design, conduct, recording and reporting studies that involve the participation of human beings stipulated by the Ministry of Health of Argentina, (Resolution Nº 1490/07) [30]. They are based in the International Declarations of Human Rights and Ethics Research (Nuremberg, 1948) [31], Helsinki treated (1964 and updates of the World Medical Asociation) [32], the Operational Guidelines for Ethics Committees (WHO 2000 - World Health Organization) [33] and the International Ethical Guidelines for Health research Involving Human Subjects (CIOMS 2017 - Council for International Organizations of Medical Sciences). [34]

Statistics

The t-test was used for statistical analysis. $p<0.001$ was considered statistically significant.

RESULTS AND DISCUSION

BNC dry weight

The dry weight of BNC per liter of King B medium (g/L) was 13.26 ± 0.41 g/L. For the preformulation and formulation studies in the laboratory, 45 g of BNC were prepared and 50 g were also prepared for the formulation of the TDDS involved in the studies of healthy volunteers.

Formulation design

(Table 1) shows the final formula of the designed TDDS. The premises taken into account for this design were: 1. Ease of self-application, for which a nano emulsion density of 1.82g/cm^3 was chosen, this allowed the dosing container to use a common cream pump, allowing the patient to apply the product themselves and avoiding resorting to a professional for the application. 2. The drying time, aiming for rapid drying, allows the effective formation of the film after the application of the TDDS. 3. Ease of washing was also a determining consideration, always keeping in mind that patient acceptability is essential when a new form of administration is introduced. On the other hand, the biodegradability of the emulsion and the atomic economy were considered, looking for the reported method for the preparation of nano emulsions, which requires the minimum possible energy expenditure. For this design, the low energy method was used. This method, unlike high intensity (energy) methods, relies on spontaneous generation of small droplets whenever the system composition experiences a phase change or inversion as a way of responding to alterations generated from changes in the composition of surfactants. Additionally, and in line with vehicle designs that minimize or replace substances derived from petroleum and other substances that cause metabolic alterations, we replace parabens with a natural preservative such as cosgard®.[35] To facilitate monitoring the controlled release of the drug from the TDDS, a pure drug model was selected. We selected the broad-spectrum antibiotic gentamicin, which allows easy monitoring of the concentration released to the acceptor solution of the in vitro delivery system. It is also a good theoretical model since gentamicin is useful for the treatment of skin infections and in the case of burn prophylaxis, the dose and route of administration are established depending on the severity of the infection, the sensitivity of the bacteria, age, weight and general condition of the patient. Taking this model as a premise, the dose was selected. [36,37] The recommended dose for patients with severe infections and normal renal function is 30 µg/kg/day administered. Taking as an example for the model, an older adult weighing 82 kg with normal kidney

function and dividing the dose into 3 applications, we find that: each TDDS should theoretically release 8.2 mg of gentamicine every 10 hours.

Homogeneity tests

When the homogeneity studies were carried out, no presence of clumps, lumps or differences in dispersion was observed in the TDDS. No phase separation was observed after centrifugation at 3500 rpm. Homogeneity was classified as very good and without phase separation. This could be due to the fact that, these are optically isotropic systems with a fine distribution of gentamicin sulfate in the particulate colloidal system.

pH measurements

When the pH was measured, a pH of 5.0 ± 0.30 was obtained. These values were reported to be in the acceptable range for the topical application formulation. In particular, in the case of TDDS, penetration into the corneal barrier is a determining factor for percutaneous absorption of the administered drug. These values can vary between 4.0 - 6.2 depending on race, age, sex and environmental factors. [30] The importance of this parameter lies in the fact that the acidic pH values of the skin surface regulate the homeostasis of the stratum corneum and the permeability of the skin barrier. The stratum corneum (SC) is formed by keratin-filled corneocytes that are surrounded by a lipophilic matrix that is composed of esters, ceramides, cholesterol and fatty acids. A slightly acidic pH helps facilitate the dissolution of the oil matrix and favors

Sephigel 305	2 g
Metacrilate polimer	0,5 g
Flax seed extract	3 mL
Glicerol	2 mL
BNC	1 g
Gentamicine Sulfate 8 mg/mL	5 mL
Cosgard type preservative	0,5 g
H_2O	e.q.f.100 mL

Table 1. Filmogenic emulsion formule and composition.

the arrival of the drug to the dermis. The dermis, in turn, is a thin, highly vascularized layer and represents the target for the penetration of the active pharmaceutical ingredient. This layer contains lymph vessels, blood vessels, sweat glands, and nerve endings.

Rheological studies

The rheological test showed that the designed semi-solid formulation presented pseudoplastic and shear thinning behavior. The measured viscosity V25ºC was 145.8±34.2 cP and V32ºC was 170.1±12.4 cP. Rheological behavior in topical formulations is influenced by factors such as pH, temperature, polymer concentrations, polymer modification, and polymer combinations. Rheological behavior of the prepared nanoemulsion is an imperative property that substantially reflects its consistency, flowability and drug release.

In vitro passive permeation studies

The in vitro release study was carried out. **(Figure 1)** shows the amount of gentamicin sulfate released (µg/cm2) during a time (h) of 10h in all the samples analyzed. The released active pharmaceutical ingredient reaches stable concentration at 4h. The graph obtained describes a direct proportionality between the accumulated fraction of drug with respect to time in a pseudosteady state, so the release of the drug defined as the mass transfer from the release system to the medium is an exponential curve. The lower concentration values obtained during the first 3 hours may be due to partial diffusion of gentamicin sulfate through a swollen matrix and hydrated pores. This is a phenomenon due to the aqueous characteristics of the formulation. As discussed in the introduction, non active pharmaceutical ingredients, have a great influence on the release process of a drug, its release rate and the concentration of the dose permeated through healthy skin. *In vitro* skin permeation studies provide data on the degree of skin penetration of the active pharmaceutical ingredient. The studies carried out allow us to support the premise that it is possible to provide a controlled release formulation that comprises a filmogenic nanoemulsion containing an active pharmaceutical ingredient immersed in an aqueous dispersion of a polymer mixture. This study made it possible to establish a stable release profile of the active agent when placed in a use environment. Furthermore, there are many problems with the influence of environmental humidity and temperature in storage stability studies of controlled release for solids patches and films. However, since it is an aqueous vehicle, which is transformed into a film by evaporation at the application site, it is possible to overcome this technological drawback. It is even possible to maintain the functionality of the product, due to the designed pharmaceutical form, even after storage for a period of time in which the formulation may be exposed to storage conditions of temperature and/or humidity elevated above the normal environmental conditions.

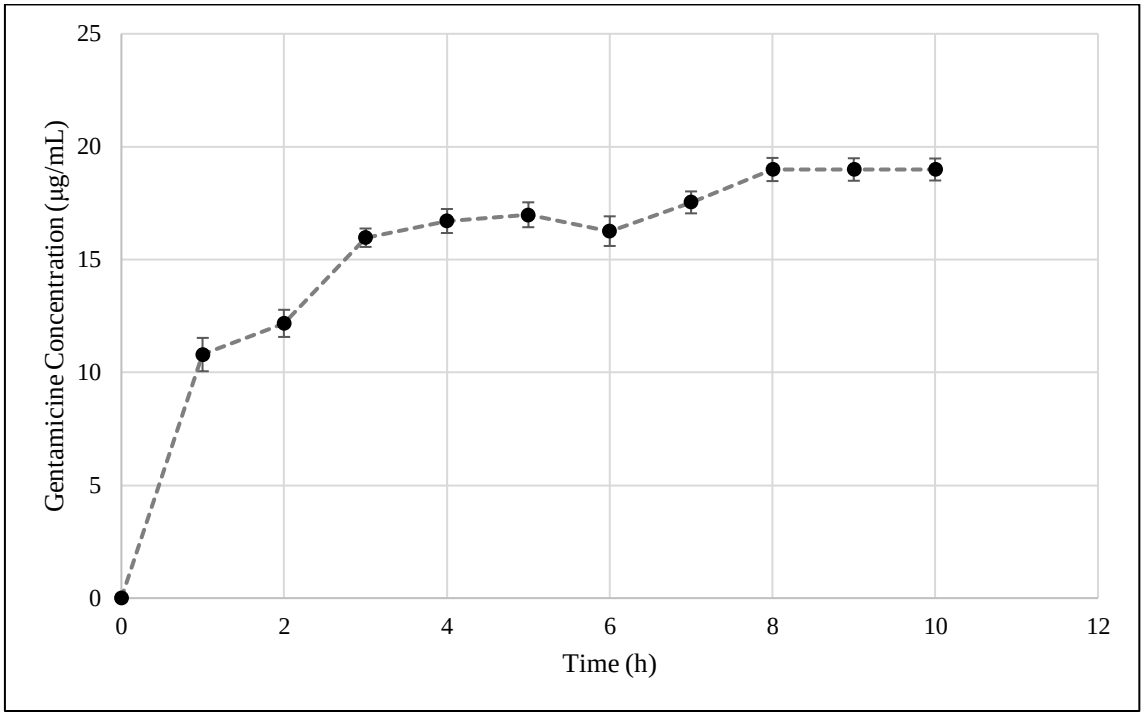

Figure 1. Cumulative amount of Gentamicine (µg/mL) during time (h) in all samples tested. The data shown represent the mean ± SD of 16 replicates.

Safety and organoleptic caracteristics acceptance studies in healthy skin volunteers.

(Table 2) shows safety studies results for 50 healthy skin voluntaries. To emulate a possible allergic development due to repeated application, we designed a double model, where the TDDS was applied in two time periods: 1h and 24h. In each case, after the application time had elapsed, the product was removed with water and the parameters were measured 1h, 24h and 48h after removing the film. Evaluated parameters were allergies represented as the appearance of edema, erythema or a feeling of dryness on the skin of the volunteers surveyed. The following categories were selected as results analised: a) absence of dermal reaction, nor edema, erythema or skin dryness b) weak reaction described as a slight presence of edema and/or erythema, skin dryness in the application area was considered. c) moderate reaction described as the presence of edema or erythema in the site of application. No allergies in the form of edema, erythema or dry skin were recorded. Previous studies carried out by other authors showed that: the use of BNC in pharmaceutical formulations presents non side effects or genotoxicity *in vitro*. [38] The absence of toxins from BNC nanofibers was demonstrated in vitro through cell viability and in flow cytometry assays on mouse cells in vivo. [39] BNC showed no adverse effects on cultured human umbilical vein endothelial cells, fibroblasts or chondrocytes. [40] *In vitro* analysis further reveals that 95% of mesenchymal stem cells accumulate in the cellulose membrane. [41] Furthermore, modified hydrophobic celluloses are GRAS materials widely used as excipients in the pharmaceutical industry. [42,43] This would explain the absence of undesirable effects in the designed formula. Organoleptics caracteristics were evaluated also. The rapid drying of the nanoemusion is a desired parameter since it increases the patient's feeling of comfort. In addition, it was reported that after the formation of the TDDS by evaporation of the nanoemulsion, the healthy volunteers did not experience a sensation of tightness in the skin and the film remained intact and wrinkles in a total of 47 volunteers. It is desired that once dry it adapts to the natural shapes of the body without cracking or wrinkling. Breakage of the TDDS was reported by 3 volunteers; this could be due to the area of skin selected for the test (back of the arm), and the patient's own activities. Ease of application of the TDDS represents a great advantage in the case of self-application proposed as one of the three fundamental objectives of the design of this TDDS. The ease of washing was also reported by the 50 healthy volunteers. This represents an advantage, since a controlled release system, water-soluble and easily removed contributes significantly to self-application and patient comfort.

Table 2: Safety studies results for 50 healthy skin voluntaries. a) No reaction; absence of dermal reaction, nor edema, erythema or skin dryness. b) weak reaction: slight presence of edema and/or erythema, skin dryness in the application area. c) moderate reaction: presence of edema or erythema in the application area. The test materials were occluded on the upper outer arms in two sets. The first set was occluded 4 hours if

Application sets	Grade of skin reaction	n=50			p value
		1 h	24 h	48 h	
4 h	No reaction	50	50	50	<0.001
	Weak reaction	0	0	0	<0.001
	Moderate reaction	0	0	0	<0.001
24 h	No reaction	50	50	50	<0.001
	Weak reaction	0	0	0	<0.001
	Moderate reaction	0	0	0	<0.001

no signs of irritation occurred the second set would be further occluded for a total of 24 hours. After the

required periods of skin contact, the patches were washed and observed for any signs of skin irritation 1 hour after removal, then 24 and 48 hours afterwards.

(Figure 2) shows the drying speed perceived by the healthy volunteer. To report the results, the perception of drying speed of the TDDS was considered and classified as: a) Fast drying speed time as less than 1 minutes; b) Medium drying speed time as 1 to 3 min; c) Slow drying speed time 3 minutes or more. Of the total healthy volunteers interviewed, 38 individuals reported a perceived drying speed of less than 1 minute, classified as fast, 13 individuals reported a perceived drying speed between 1 and 3 minutes, classified as medium perceived speed and only 1 of the participating individuals reported perceived drying speed greater than three minutes, classifying it as slow. This could be due to skin factors such as pH, race, hormonal status, oiliness or normal dryness of the patient's skin type. However, a drying speed perceived as fast was reported by 76% of healthy volunteers.

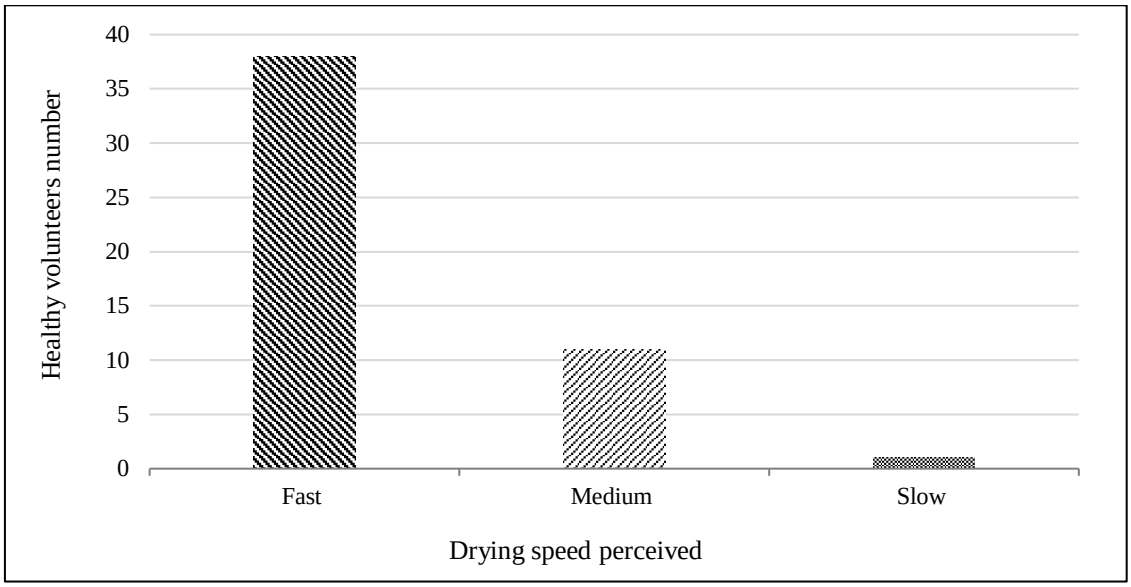

Figure 2: Organoleptic characteristics analysis. Perceived drying speed by the healthy volunteer was considered as: a) fast drying time: less than 1 minutes, b) medium drying time: 1 to 3 min, c) slow drying time: 3 minutes or more.

CONCLUSIONS

The objectives of this work were achieved. The successful design of a TDDS based on BNC as a copolymer allowed efficient controlled release from the film formed by *in situ* drying of the nanoemulsion. The designed TDDS meets the criteria for adequate release of the active ingredient in a pure drug model. The nanoemulsion quickly forms a film that adequately adheres to the skin tissue without breaks and wrinkles. In the study carried out on 50 healthy volunteers, the absence of edema, erythema and dry skin was confirmed in two models. One of them with application of TDDS for 24 hours, demonstrating that sustained or chronic application also presents an absence of allergic reactions. Technologically, the potential in the conservation of TDDS stands out, which lies in the design of a fast-drying filmogenic naoemulsion, which prevents deterioration on the shelf due to environmental humidity and high temperatures. The premises of ease of self-application, controlled release and ease of handling were verified in the tests carried out on healthy volunteers. It is possible to avoid the unwanted effects of other forms of administration such as

oral, percutaneous and intravenous routes by applying TDDS. Among the most significant characteristics of this formulation design are: its non-invasive nature, the increase in the bioavailability of the drug, the ease of application, the absence of gastrointestinal side effects, its accessibility to the patient, its painless nature, bypassing the hepatic portal system and drug metabolization. More studies are needed on pure drug models, mixtures and *in vitro* release profiles. However, the incorporation of NBC into the TDDS formula opens an opportunity for a wide variety of drugs whose transdermal application would improve the patient's quality of life, which is the fundamental premise of all available pharmaceutical technology.

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