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Research paper

Use of photoacoustic spectroscopy in the characterization of inclusion complexes of benzophenone-3-hydroxypropyl-β-cyclodextrin and *ex vivo* evaluation of the percutaneous penetration of sunscreen

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ABSTRACT

This work is aimed to evaluate the application of photoacoustic spectroscopy (PAS) in the characterization of inclusion complexes of benzophenone-3 (BZ-3) and hydroxypropyl-β-cyclodextrin (HPCD) and to analyze the *ex vivo* percutaneous penetration of sunscreens and their reaction with the skin. The formation of inclusion complexes of BZ-3 and HPCD was performed by co-precipitation in stoichiometric ratios of 1:1 and 1:2. Thermal analysis and PAS characterized these inclusion complexes, and they indicated that the stoichiometric ratio of 1:2 was best. Sunscreen formulations were prepared and applied on the ears of rabbits. PAS suggested that the formulation with the complex resulted in lower penetration of BZ-3. Histological analysis demonstrated that the use of the formulation with BZ-3 was associated with an increase in the comedogenic effect and the presence of acanthosis, while no such effect was found in the formulation with the complex. The formulation with the BZ-3-HPCD complex is a promising strategy for improving the photoprotective effect of BZ-3. PAS can be used in the study of inclusion complexes with cyclodextrins and the evaluation of the percutaneous penetration of sunscreen formulations. Further tests are being conducted using PAS to monitor *in vivo* changes in the optical absorption spectra of formulations and to investigate their photostability.

1. Introduction

Each year, between 2 and 3 million non-melanoma skin cancers and over 130,000 melanomas are diagnosed worldwide. The overall incidence of melanoma continues to increase, but the main factors that predispose to the development of melanoma continue to be sun exposure and sunburn [1].

Concerns about the consequences of exposure to UV radiation and its correlation with cancer development have triggered a public education campaign promoting the use of sunscreens. The impact of this campaign has been reinforced by concerns over the destruction of atmospheric ozone, which has increased the amount of UVB radiation that reaches Earth's surface [2]. An estimated 10% decrease in ozone levels will result in an additional 300,000 new cases of non-melanoma skin cancer and 4500 cases of melanoma [1].

Sunscreens should have minimal absorption through the skin because their penetration can cause phototoxic reactions and photoallergic reactions [3]. The solar UV filters present in sunscreens are designed to absorb, reflect, or refract ultraviolet radiation, and thereby protect the skin against harmful effects caused by exposure to sunlight. Their effectiveness depends on the integrity of the skin to prevent the sunscreen from penetrating the transdermal systemic circulation [4–6].

Among the many organic UV filters, benzophenone-3 (BZ-3) is a component that is thoroughly characterized, relatively inexpensive, and capable of absorbing UVA (320–400 nm) and UVB (280–320 mm) [4,7–9]. Its maximum permissible concentration in formulations is 6% [1,10]. The transdermal absorption of BZ-3 in humans may reach 2%, as it is able to permeate the skin and reach the bloodstream after topical application [11]. Studies have detected BZ-3 in human urine [7,10,12], breast milk [5], and blood plasma [10,11], and they have shown a high incidence of photoder-

Abbreviations: PAS, photoacoustic spectroscopy; BZ-3, benzophenone-3; HPCD, hydroxypropyl-β-cyclodextrin; CDs, cyclodextrins; β-CD, beta-cyclodextrin; BZ-3-HPCD, inclusion complex benzophenone-3-hydroxypropyl-β-cyclodextrin.

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matitis among BZ-3 users [3,8]. In addition, BZ-3 is solid at room temperature but slightly soluble in cosmetic preparations, such as lotions and creams. It adds an undesirable color to cosmetic products, and it is unstable in the presence of light and heat [13]. The use of different vehicles or nanoparticles, increasing the viscosity of the formulation, and complexation with cyclodextrins (CDs) are strategies for reducing the systemic penetration of sunscreen [5,14]. In drug delivery systems used on skin, CDs have been reported to improve the dispersion of drugs and influence their skin permeation rates [15]. In addition, they can be used to stabilize emulsion systems by complexation of fatty acid residues of the oil phase. The results showed that in particular the cyclodextrins seemed to induce fundamental changes in formulation microstructure.

To this end, the use of CDs has increased, especially β-cyclodextrin (β-CD). β-CD has a hydrophilic surface and hydrophobic central cavity and thus provides an environment less polar than water, giving it the ability to form inclusion complexes with molecules of apolar character. Due to the low aqueous solubility of β-CD (18.5 mg/mL at 25 °C), some chemically modified CDs, such as hydroxypropyl-β-cyclodextrin (HPCD), have gained importance due to their improved solubility (>600 mg/mL) [15,16]. Studies have shown that the complexation of HPCD significantly increased the solubility and photostability of sunscreens [12] and significantly reduced the release and penetration of BZ-3 through the biological membrane, without suppressing the properties of UV absorption [17].

The skin has a complex structure consisting of heterogeneous layers presenting several routes and mechanisms for the penetration and interactions of substances. In addition, it is continuously exposed to environmental changes like different conditions of humidity, temperature, radiation, and the contact with microorganisms and toxins [18–20]. These skin working condition and their structural characteristics have evidenced how troublesome is the development of simple models to predict the processes involved in the penetration and interaction of substances [18,19]. As a consequence, a great number of techniques have been developed in order to provide a better understanding of these processes, especially for *in vivo* non-invasive measurements that usually maintain the skin physiological characteristics, what may not occur when wet standard chemistry methods are used. Also, *in vivo* direct measurement provides information not accessible to conventional techniques (*in vitro*), such as intermolecular energy transfer or energy storage processes, feature components inside cells, and photo-thermal dissipation [21].

In this way, spectroscopic methods such as Confocal Raman Spectroscopy [22] and Fourier Transform Infrared Spectroscopy (FTIR and ATR) [23–25] have been successfully used by exploring the finger print characteristic of the infrared optical absorption bands. The photothermal methods like Photothermal Radiometry [26], Photothermal deflection [20,27], FTIR-PAS [27–29], and UV–vis photoacoustic spectroscopy [30–33] have also proven to be important techniques for analyzing and evaluating the penetration and distribution of substances through the skin. They are based on the detection of the sample absorbed energy that is converted into heat, being therefore complementary to pure optical procedures. They are non-destructive techniques of reasonable low cost with special advantages for studies in opaque samples and to perform depth profile analyses. Certainly, the choice of a specific method depends on availability, wavelength selectivity according to the investigated formulation, study protocol, and sampling conditions. In this context, UV–vis photoacoustic spectroscopy (PAS) has been shown to be important for sunscreen measurements providing the formulation optical absorption bands in the UVA and UVB spectral regions, essential for sunlight protection [31]. Several *ex vivo*, *in vitro*, and *in vivo* studies have used this technique to determine the penetration of formulations for topical application [30–33].

Despite previous studies on the inclusion complex benzophenone-3-cyclodextrin (BZ-3-CD), to the best of our knowledge, there have been no reports on the use of photoacoustic spectroscopy to assess this complex and its percutaneous penetration.

Whereas BZ-3 is the most widely employed UV filter in sunscreen formulations, this work aimed to use PAS and thermal analysis to characterize the inclusion complex of BZ-3 with HPCD, to use PAS to analyze the *ex vivo* percutaneous penetration of sunscreens, and to evaluate histologically the reaction of the formulations with the skin.

2. Materials and methods

2.1. Determination of the specific molar absorbance of benzophenone-3 (BZ-3)

We weighed 0.01 g of BZ-3 (MRP Indústria de Óleos Vegetais – Bofete, Brazil) and dissolved it in 100 mL of a 60:40 (v/v) ethanol–water mixture. The solution was kept under agitation at 150 rpm (in a cooled incubator with stirring, Tecnal – TE 424, Piracicaba, Brazil), protected from light at 37 ± 1 °C for 2 h, filtered through a Millipore 0.45 μm membrane, and diluted with distilled water to the following proportions: 1:2, 1:3, 1:4, 1:5, 1:7, 1:10, and 1:20. The reading of absorbance was performed on a spectrophotometer (Varian Cary 50 UV–visible, Santa Clara, USA), which was set to 291 nm. A straight line was fitted to the data, and its slope generated the specific molar absorbance (ε) of the BZ-3.

2.2. Studies of the phase solubility

Solubility analysis followed the methodology of Higuchi and Connors [34]. An excess of BZ-3 present in a solution of ethanol–water (60:40, v/v) was added to aqueous solutions containing different concentrations (0–14 mM) of HPCD (Sigma–Aldrich Chemie, Steinheim, Germany). The suspensions were shaken in capped bottles at a temperature of 25 ± 1 °C, protected from light, and shaken at 150 rpm for 3 days. After this period of time, the contents of each bottle were filtered using Millipore 0.45 μm membranes and analyzed by high-performance liquid chromatography (HPLC) to determine the concentration of BZ-3. A liquid chromatography (Varian ProStar, Santa Clara, USA), which was equipped with pump (model 240), manual injector and a photodiode detector (model 330) set at 291 nm, was used. Analyses were performed at room temperature on a reversed phase column (Ods C18 Hypersil, 150 mm × 2.1 mm, particle diameter 3 μm, and pore size 120 Å, Thermo, Waltham, USA). The sample injection volume was 20 μL with a mobile phase of acetonitrile and water (70:30, v/v) and a flow of 0.4 mL/min. The data were analyzed in triplicate. The solubility diagram was plotted between the molar concentration of BZ-3 in solution and the molar concentration of HPCD. The values of stability constant (K) were calculated with Eq. (1) [34]:

$$K = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (1)$$

In this equation, S_0 is the solubility of BZ-3 in the absence of HPCD and the slope was obtained by means of phase solubility diagram.

2.3. Preparation of inclusion complexes

Inclusion complexes were prepared by the co-precipitation method in which the guest molecule or its solution was added slowly with stirring to the aqueous solution of CD [16,34]. To obtain the BZ-3-HPCD inclusion complex, the BZ-3 dissolved in

20 mL of ethyl alcohol was added to the solution of HPCD in 30 mL of water in a molar quantity corresponding to stoichiometric ratios of 1:1 and 1:2. The mixture was maintained at 150 rpm for 120 h at 37 °C and protected from light. Next, the mixture was filtered through a Millipore 0.45 μm membrane and submitted to a rotary evaporator at 40 °C under a vacuum. The BZ-3 concentration of inclusion complexes in stoichiometric ratios of 1:1 and 1:2 was determined by HPLC after proper dilution.

2.4. Differential scanning calorimetry (DSC) and thermogravimetry (TG)

Samples of BZ-3, HPCD, and BZ-3-HPCD inclusion complexes in stoichiometric ratios of 1:1 and 1:2 were weighed, placed in platinum capsules, and submitted to simultaneous analysis of DSC and TG. Equipment STA 409 PG (Luxx)-NETZSCH (Selb, Germany) was used in a temperature range of 25–500 °C, with a heating rate of 10 °C/min under nitrogen atmosphere (20 mL/min).

2.5. Preparation of sunscreen formulations

Sunscreen was prepared by a standard formulation (base) consisting of Eumulgin® HRE-40, 8% (Saint Fargeau Ponthierry, France), Acculyn®-33, 10% (La Mirada, USA), triethanolamine, 2% (San Francisco, USA), and water in sufficient quantity to make 10 g of product, to which BZ-3 (4%) was added.

2.6. Animal model studies

Adult male albino New Zealand rabbits, weighing around 2.9 kg, were used. They were maintained at 20 °C in individual cages for 15 days at the Experimental Farm of the State University of Maringá (Maringá, PR, Brazil) on a 12 h light/dark cycle with water and food (Nuvital®, Colombo, Brazil) *ad libitum*. The animals ($n = 18$) were divided into four groups, and the concave side of their right ears, considered the control, was treated with distilled water. The concave side of the left ears was treated with formulations. The applications were performed once a day, early in the morning at the same time each day. Group A (three animals) was treated with distilled water in both ears, group B (three animals) received the base formulation, group C (6 animals) received the formulation containing BZ-3, and group D (6 animals) received the formulation containing the BZ-3-HPCD inclusion complex. A systematic macroscopic evaluation was performed daily to check for possible occurrence of erythema, edema, desquamation, presence of comedones, and inflammatory reactions. This study was approved by the Animal Ethics Committee of the State University of Maringá, under the Protocol CEAE No. 050/2008.

On the 15th day, 30 min after application of the formulations, the animals were killed by overdose of thionembutal (Abbott Laboratories, North Chicago, IL, USA). The ears were removed and the percutaneous penetration measures were taken after removing the skin, resulting in samples with thicknesses (l_s) ranging from 350 to 450 μm. The final sample was composed of the stratum corneum, the epidermis, and dermis. With this procedure, the photoacoustic experiments were performed about 20 min after animal killing.

2.7. Optical absorption evaluation of the formulations using photoacoustic spectroscopy (PAS)

Initially, the absorption spectra were obtained from the sample HPCD, BZ-3, and BZ-3-HPCD inclusion complexes in the stoichiometric ratios of 1:1 and 1:2 at the ultraviolet and visible wavelengths (between 240 and 500 nm). Later, tests were conducted with the base formulation, the formulation with BZ-3, and the formulation with the BZ-3-HPCD inclusion complex in the stoichiometric ratio of 1:2.

In this study, the PAS measurements were performed using a custom-built experimental setup, shown in Fig. 1A. This technique refers to the ability of photoacoustic spectroscopy to analyze depth penetration profile, which is specified by the thermal diffusion length, $\mu = [\alpha/(\pi f)]^{1/2}$, where α is the sample thermal diffusivity and f is the light modulation frequency, as well as light penetration depth ($l_\beta(\lambda) = 1/\beta(\lambda)$), with β as the optical absorption coefficient and λ the light wavelength [30].

The monochromatic light was obtained from a 1000 W xenon arc lamp (model 68820; Oriel Corporation, Irvine, USA) and a monochromator (model 77250; Oriel Corporation, Irvine, USA). The light beam was modulated with a mechanical chopper (Stanford Research Systems SR540, Sunnyvale, USA). The photoacoustic cell was custom-designed to have a minimum volume. Made from an aluminum block, it was machined to hold samples with maximum dimensions of about 5 mm in diameter and 1 mm in thickness, and it allowed light to enter through a highly transparent quartz window 6 mm in diameter and 2 mm thick. The microphone chamber was 15 mm from the cell and connected to the sample-holder chamber by means of a 1-mm-diameter duct. The capacitive microphone was a highly sensitive 12 mm diameter (Brüel & Kjaer model 2639, Naerum, Denmark) which provides a high gain of 50 mV/Pa and flat performance for frequency response from 1 Hz to 10 kHz. The lock-in amplifier was from EG & G Instruments (model 5110, Oak Ridge, USA). To obtain the photoacoustic spectra, the light modulation frequency was set at 22 Hz for measurements on the external surface of the skin and at 22 and 38 Hz when the lighting was on the inner surface of the skin. Measurements were recorded between 240 and 500 nm. Data acquisition were done with a personal computer, and the PAS spectra were normalized with respect to the carbon black signal [30].

In the photoacoustic measurements, the thermal diffusion length (μ) provides the sample skin depth, which contributes to the photoacoustic signal [30–33]. To evaluate μ, α should be known in advance. With low frequency modulation, it is possible to examine deeper layers beneath the skin surface, while higher frequencies allow the assessment of the skin surface, as illustrated in Fig. 1B. For a good review on the subject, we refer to the work of Wartewig and Neubert [29]. Considering that the light modulation frequency was 22 Hz and the thermal diffusivity of the skin was $\alpha = 4.0 \times 10^{-4}$ cm^2 s^{-1} [35], the thermal diffusion length μ in our measurements can be estimated as 24 μm.

To guarantee the detection of the active substance propagated through the skin thickness, the sample was excited first onto the epidermal side (the sample external face), i.e., the side on which the formulations were applied as shown in Fig. 1C, and then on the dermal side (the sample internal face). Thus, considering that the skin sample thickness ranged between 350 and 450 μm, the formulations' optical absorption bands detected at the dermal side of the sample means that applied substances propagated throughout the skin.

2.8. Macroscopic and histological evaluation of topical sunscreen formulation

Samples with an area on the order of 3 cm^2 were removed from each ear and fixed in Bouin solution. After 12 h, the samples were dehydrated by incrementally increasing the concentration of ethylic alcohol, cleared in xylol, included in paraffin, and cut into 5-μm-thick histological sections, which were stained with hematoxylin-eosin. The histological analysis was performed to determine the acanthosis, the comedogenic potential, and the presence of inflammatory reaction in the samples. The comedogenic potential was determined according to the protocol of EVIC-CEBA Laborato-

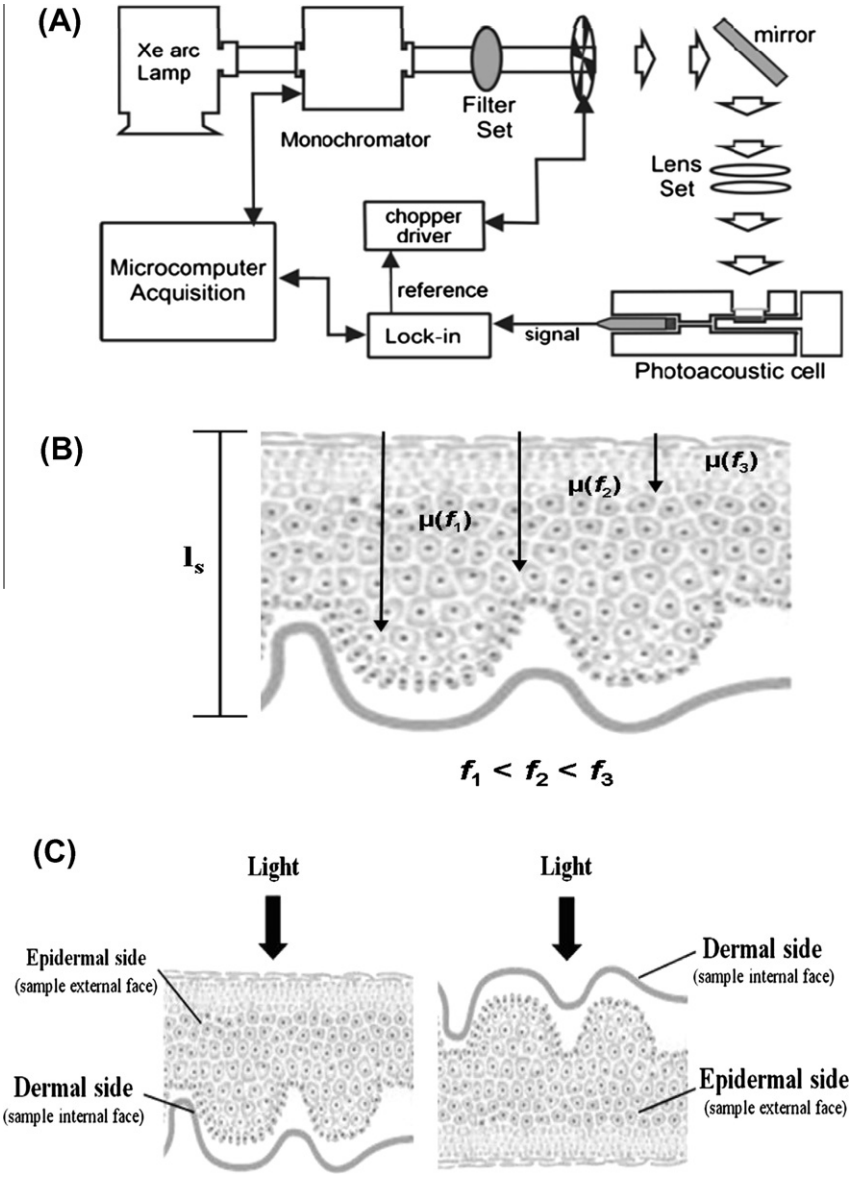

Fig. 1. (A) Experimental setup of photoacoustic spectroscopy analysis. (B) Arrows illustrating μ for different frequencies in the photoacoustic measurement that is related to the absorbing substance depth penetration in the sample. (C) Representation of the incident light into the epidermal or dermal skin sides, respectively, to obtain the optical absorption spectrum using the photoacoustic method.

rie de Recherche et d'Experimentation by Fulton et al. [36] and Kligman and Kwong [37]. The comedones' number and length were evaluated in six skin histological sections per animal. The comedones were measured on an Olympus BX40 microscope (Center Valley, USA) at the 10× ocular objective, containing a micrometer disc. The comedones were divided, according to their length, into three different groups: small (<400 μm), medium (400–850 μm), and large (>850 μm). The comedones of each group were counted and multiplied by factors of 0.5, 2, and 5 for the small, medium, and large length groups, respectively. After summation of the results of the three groups, the comedogenic potential of each ear was classified as none (value < 10), mild (10 < value ⩽ 30), moderate (30 < value ⩽ 90), and severe (value > 90) [36,37].

Data from the control group and the three groups treated with different formulations were submitted to an analysis of variance (ANOVA), and a nonparametric Kruskal–Wallis test was also used to compare groups. The significance level was 5% using the software Statistica 8.0/2008 (Stat Soft, Inc., Tulsa, USA).

3. Results and discussion

3.1. Characterization of the BZ-3-HPCD complex

Fig. 2 shows the data for absorbance at 291 nm of the BZ-3 solution in an ethanol–water mixture. The value calculated for the specific molar absorbance (ε) of the BZ-3 was 15,086 L mol^{-1} cm^{-1}, obtained from the slope of the line fitted to the data of absorption as a function of the concentration of BZ-3.

The phase solubility diagram, in which the solubilized BZ-3 concentration was plotted as a function of the concentration of HPCD, is shown in Fig. 3.

The addition of HPCD significantly increased BZ-3's aqueous solubility. Furthermore, a linear relationship was observed between the solubility of BZ-3 and the concentration of HPCD. A similar result was found by other researchers [5,38–40].

Based on the phase solubility diagram shown in Fig. 3, the inclusion of BZ-3 by HPCD was considered, according to the theory of

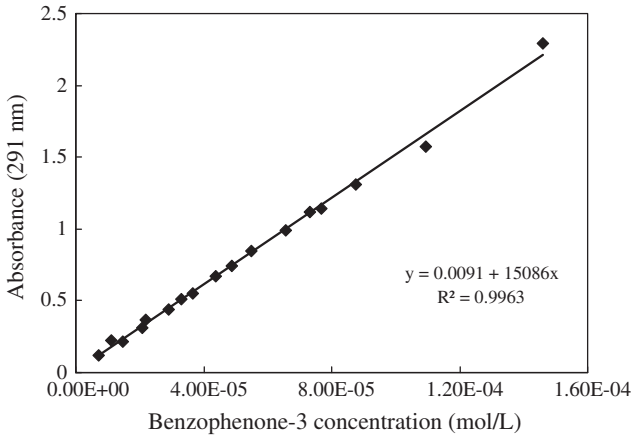

Fig. 2. Specific molar absorbance of benzophenone-3 (BZ-3).

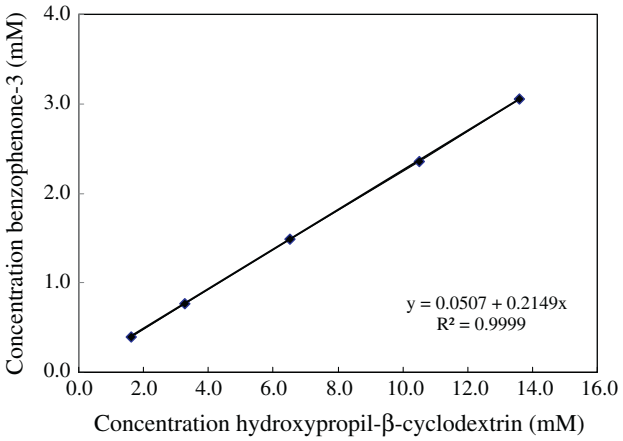

Fig. 3. Influence of hydroxypropyl-β-cyclodextrin concentration (HPCD) on the aqueous solubility of benzophenone-3 (BZ-3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Higuchi and Connors [34], a phenomenon of the A_L type, suggesting a reaction in the stoichiometric ratio of 1:1. The stability constant ($K_{1:1}$) for the complexation was estimated at 5398 M^{-1}. Sarveiya et al. [12] reached a stability constant very similar to ours ($K_{1:1}$ = 5839 M^{-1}).

The methodologies of DSC and TG were used simultaneously for the preliminary characterization and quantification of inclusion complexes with BZ-3 and HPCD (Fig. 4). In general, the complexation is verified by the disappearance of the endothermic peak characteristic of the guest molecule inside the CD [41,42]. In the HPCD analysis, an endothermic peak was observed at 75.8 °C, corresponding to water loss. Above 300 °C, irregular peaks appear, which are related to the decomposition of HPCD [41,43]. BZ-3 showed an endothermic process with a melting point around 68.5 °C. The other endothermic peak, 310 °C, indicates the degradation point of this compound [14]. Analyzing the DSC curves at temperatures between 180 and 310 °C revealed that the complex evaporation peak decreases when compared with pure BZ-3, suggesting the formation of complexes (Fig. 4A).

In the BZ-3-HPCD inclusion complex, at the stoichiometric ratio 1:1, an endothermic peak was observed at temperature of 68 °C, in which there is BZ-3 available (Fig. 4A). This peak also corresponds to the melting point of pure BZ-3, demonstrating the presence of this free substance in the complex, a possibility that can be corroborated by the modest degradation of this complex in the spectrum

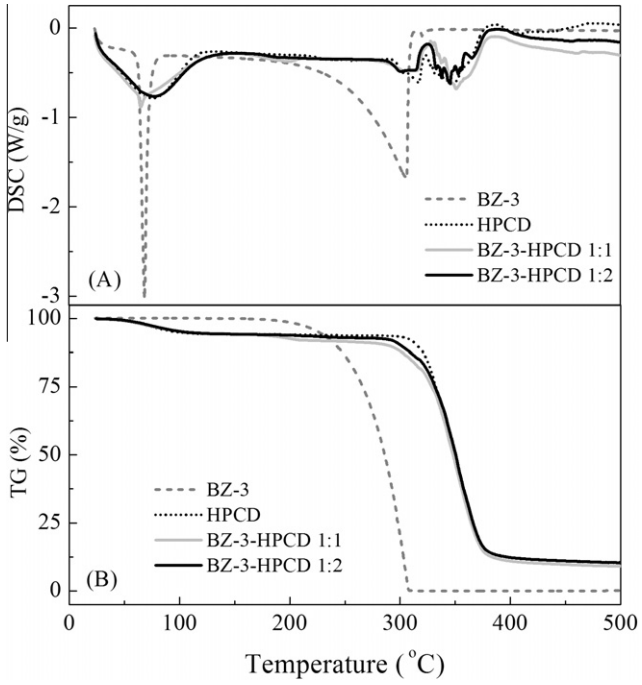

Fig. 4. Thermal analysis of (A) DSC curves and (B) TG curves for benzophenone-3 (BZ-3), hydroxypropyl-β-cyclodextrin (HPCD), and benzophenone-3-hydroxypropyl-β-cyclodextrin inclusion complexes (BZ-3-HPCD) in the stoichiometric ratio of 1:1 and 1:2.

region near 200 °C. This description was also confirmed in TG, which verified the beginning of free BZ-3 loss and the BZ-3-HPCD complex in a stoichiometric ratio of 1:1 at 200 °C (Fig. 4B).

The BZ-3-HPCD complex in the stoichiometric ratio of 1:2 was similar to the spectrum of pure HPCD. It did not show any transition at the melting point, giving evidence that BZ-3 was complexed in the cavity of HPCD (Fig. 4A). At 200 °C, it was observed that the BZ-3-HPCD complex in the stoichiometric ratio of 1:2 also showed similar behavior to that of pure HPCD. However, the spectrum of the BZ-3-HPCD complex in the stoichiometric ratio of 1:1 was more similar to the behavior of pure BZ-3, suggesting an inferior complexation compared with the BZ-3-HPCD complex in the stoichiometric ratio of 1:2. Another situation that suggests the stoichiometric ratio of 1:2 is better than 1:1 is that the BZ-3-HPCD complex in the stoichiometric ratio of 1:2 exhibits more degradation at higher temperatures (300 °C) than the stoichiometric ratio of 1:1 (250 °C) (Fig. 4A).

Al-Rawashdeh et al. [44] studied the inclusion complexes of BZ-3, octocrylene, and ethyl-hexyl-methoxy-cinnamate with HPCD aqueous solutions and solid phases. The formation of inclusion complexes was confirmed experimentally by DSC, scanning electron microscopy (SEM), and C-13-NMR methodologies. The photodegradation reaction was also explored using UV–vis spectrophotometry and high-performance liquid chromatography (HPLC), and the results indicated that complexation with HPCD has the potential to increase the sunscreen's photostability in solution.

3.2. Photoacoustic spectroscopy (PAS)

The first step of PAS involved measuring the optical absorption spectra of the active agent (BZ-3), the complexing agent (HPCD), and the possible inclusion complexes (BZ-3-HPCD) to evaluate the formation of complexes and to determine the optimal stoichiometric ratio for better preparation of sunscreen formulations.

The presence of a large absorption band in the ultraviolet region, between 200 and 400 nm, demonstrated that BZ-3 and the complexes exhibited a high absorption coefficient in the spectral regions from UVC to UVA (Fig. 5A). The absorption spectrum suggested the inclusion complex formation of BZ-3 with HPCD and the stoichiometric ratio of 1:2 was associated with the lowest intensity of BZ-3. Therefore, considering this result, the sunscreen formulation was prepared from the complexation of BZ-3 with HPCD in the ratio 1:2 and added to the base.

Fig. 5B shows the spectra of the three types of formulations tested: pure base, base with BZ-3, and base with complex. The absence of absorption in the pure base allowed the evaluation of the spectra of samples prepared with the active principles. The spectra showed that, in the formulation of the base with BZ-3, the band with maximum absorption at about 280 nm did not appear in the sample of the base with complex, again providing evidence that there was complexation between the BZ-3 and HPCD.

The CDs can be used to stabilize emulsion systems by complexing with fatty acid residues of the oil phase, improve the dispersion of substances, and influence their skin permeation rates [15,45]. Considering that BZ-3 is solid at room temperature and has limited solubility in water [13], the cosmetic preparations are commonly prepared using hydrophobic base containing about 20% of lipophilic agent. Due to the need of comparison between the formulations used in this research, a nonionic O/W emulsifier (Eumulgin® HRE-40) was used at a concentration of 8%. In practice, it was observed that in preparing the formulation with BZ-3-HPCD complex did not need of the nonionic O/W emulsifier, indicating that this formulation overcomes the limited solubility of BZ-3 and is a strategy for reducing the systemic penetration of sunscreen. Klang et al. [45] showed that the CDs seemed to induce fundamental changes in formulation microstructure, and new surface active molecule complexes were formed which lower interfacial tension and stabilize the systems. These amphiphilic molecules formed represent eudermic emulsifying agents, in which the CDs themselves cannot permeate through the skin due to their large molecular weight.

In addition, it is important to mention that in formulations with BZ-3, a yellow coloration is known to occur that imparts undesirable color to cosmetic products, as previously described by other authors [7,13], however, this coloration did not occur in the formulation with BZ-3-HPCD complex, showing that this formulation does not influence the appearance of the final product.

The next step was the measurement of the skin samples of the animals treated with the topically applied formulations. First, PAS was obtained from the sample that was excited on the epidermal side (on which the formulations were applied) using the light modulation at 22 Hz, and after PAS was obtained from the sample excited on the dermal side using light modulation at 22 Hz and at 38 Hz (Fig. 6).

The spectra of substances that form the complex (Fig. 5A) were used to identify these substances in the skin (Fig. 6). The band featuring HPCD (approximately 276 nm) was also found in the skin, making it difficult to identify because the skin absorbs radiation in this wavelength. Between the wavelengths of 325 and 380 nm, no optical absorption of the skin and HPCD was observed, suggesting that BZ-3 was responsible for the optical absorption in this spectral range. The optical absorption band around 410 nm was associated with blood present in the area that was excited.

In Fig. 7, the photoacoustic spectra of optical absorption from the rabbit skin with different formulations were compared. The results of excitation on the epidermal side were obtained at a frequency of 22 Hz (Fig. 7A). As mentioned before, with this procedure, the thermal diffusion length (μ) in the skin contributing to the generation of the photoacoustic signal is on the order of 24 μm. Fig. 7B and C shows the effects evaluated at the dermal side of skin. For example, considering a sample 400 μm thick and used frequencies to obtain the spectra at 22 and at 38 Hz, the corresponding penetration depths below the surface of the skin were around 376 and 382 μm, respectively. The penetration of the formulation through the skin, when compared with the application of only water, was checked with the analysis of photoacoustic spectra. On the dermal side, it was observed that penetration decreases with increasing frequency. In the curve with a frequency of light modulation of 38 Hz (i.e., 382 μm in depth), the penetration was lower than that of 22 Hz (this measure was performed on the side to which the formulation was not applied).

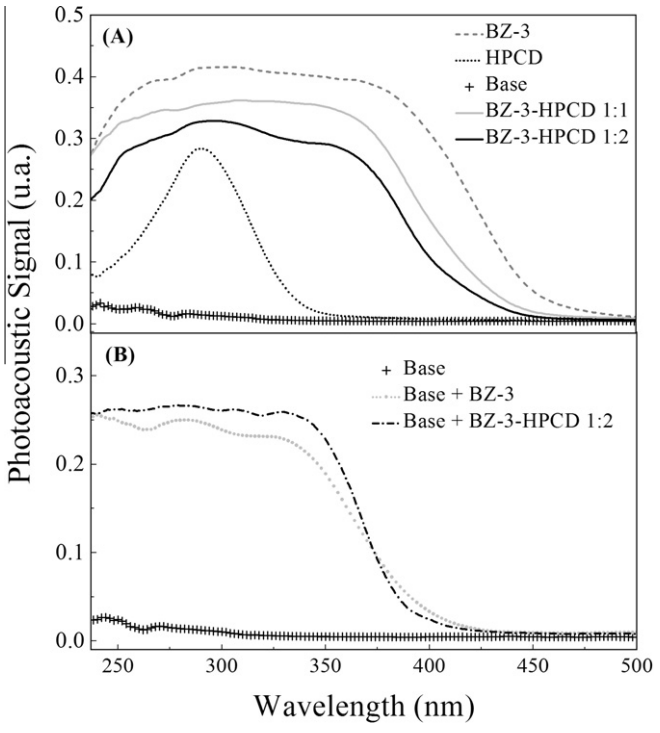

Fig. 5. Photoacoustic spectra of UV absorption of the pure substances and cosmetic formulations of: (A) hydroxypropyl-β-cyclodextrin (HPCD), benzophenone-3 (BZ-3), benzophenone-3-hydroxypropyl-β-cyclodextrin inclusion complex (BZ-3-HPCD) in the stoichiometric ratio of 1:1, benzophenone-3-hydroxypropyl-β-cyclodextrin inclusion complex (BZ-3-HPCD) in the stoichiometric ratio of 1:2, base, and (B) base, base + BZ-3, base + BZ-3-HPCD in the stoichiometric ratio of 1:2.

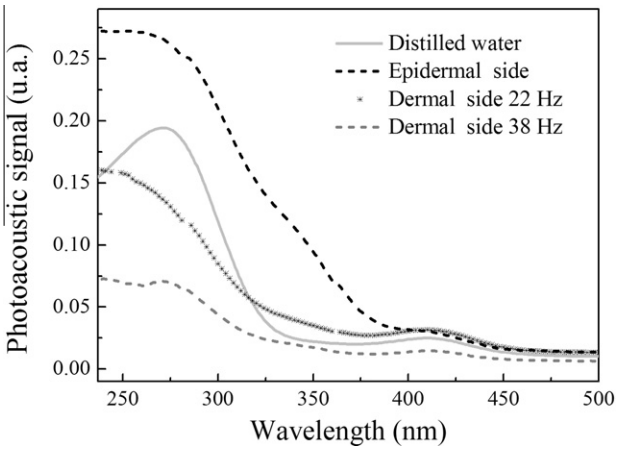

Fig. 6. Absorption spectra obtained by PAS, conducted on the frequencies of 22 and 38 Hz, on the epidermal and dermal sides of the rabbit ears treated with distilled water (control) and base + benzophenone-3-hydroxypropyl-β-cyclodextrin inclusion complex (BZ-3-HPCD) in the stoichiometric ratio of 1:2.

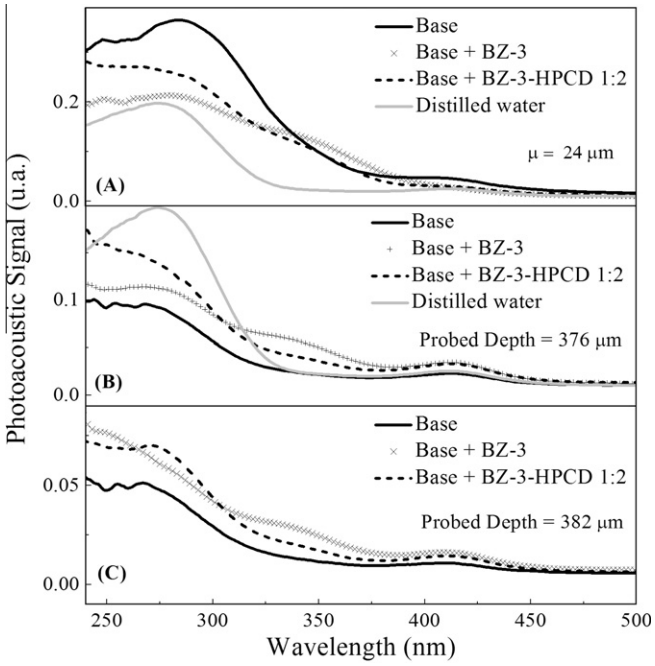

Fig. 7. Photoacoustic spectra of the skins of rabbit ears (A) carried out at a frequency of 22 Hz (24 μm deep) on the epidermal side after application of formulations, (B) at a frequency of 22 Hz (penetration depth of about 376 μm) on the dermal side and (C) at a frequency of 38 Hz (penetration depth of about 382 μm) on the dermal side.

Thus, the analysis of results occurred in a region more superficial on the dermal side and deeper in relation to the epidermal side. In the ears that were treated with the formulation with complex, it was possible to conclude that there was not a high penetration rate of BZ-3 in the deeper regions of the skin compared with the outermost layer. Previous studies have also shown that complexation with HPCD significantly reduced the release and penetration of BZ-3 through the skin [17]. Probably, the explanation for this may be the lipophilic character of the skin which is difficult for the propagation of hydrophilic compounds throughout its structure. Hadgraft [46] emphasized that the predominant penetration route is through the intercellular spaces that contain structured lipids and a diffusing molecule has to cross a variety of lipophilic and hydrophilic domains before it reaches the junction between the stratum corneum and the viable epidermis. Saino et al. [47] studied the skin permeation and distribution of ibuprofen by using nanostructures (coagels) and concluded that the permeation of substances into the skin depends on their release from the formulation, their penetration into the stratum corneum, and their diffusion into the skin layers to the dermis to reach the circulation.

It was found that the base reproduces the spectrum of skin in the control to which distilled water was applied. Measurements performed on the dermal side showed that in the spectral region of interest for sunscreens (300–400 nm), the formulation with the BZ-3-HPCD complex permeated less than that containing only the BZ-3, and the stoichiometric ratio of 1:2 has proved to be the most viable alternative to reduce the penetration of BZ-3 through the skin. The penetration rates of three different sunscreens were evaluated *in vivo* by Sehn et al. [33] using PAS and a group of 15 healthy white skin volunteers. The results demonstrated the ability of the technique to discriminate the propagation time of the products investigated, confirming its sensitivity to measure the penetration rate topically applied substances through skin.

In addition, it is important to mention that previous studies have shown that the complexation with HPCD significantly increased the sunscreens photostability [12,48]. For example, Yang et al. [48] evaluated the avobenzone photostability complexed with HPCD *in vivo* and found that the presence of HPCD (30% w/w) in the formulation imparted photoprotective efficiency, as evidenced by lower levels of sunburn and skin edema, indicating that complexation with HPCD enhances the photoprotective effect of sunscreens. Thus, considering that PAS is an important strategy for *in vivo* studies, we are awaiting approval by the Ethics Committee to use this method for photostability testing, especially for *in vivo* measurements monitoring the possible changes in the formulations optical absorption spectra.

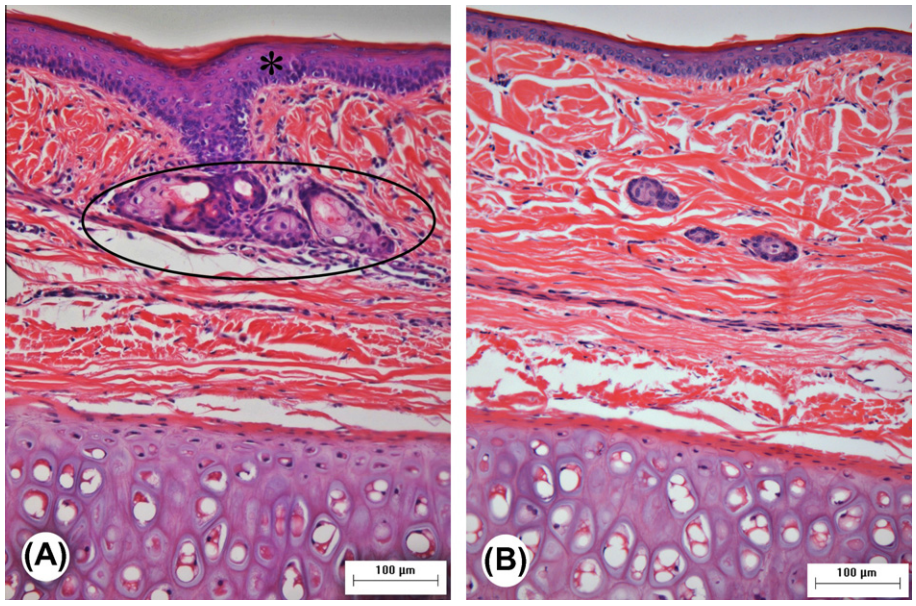

Fig. 8. Histological sections of rabbit ears showing different types of comedones and acanthosis. (A) Average comedones + acanthosis (*); (B) small comedones. The bar length is 100 μm (hematoxylin-eosin stain). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Histological evaluation of rabbits ears treated with water (control; $n = 3$/group), with base formulation ($n = 3$/group), formulation with benzophenone-3 (BZ-3) ($n = 6$/group), and formulation with benzophenone-3-hydroxypropyl-β-cyclodextrin (BZ-3-HPCD) in the stoichiometric ratio of 1:2 ($n = 6$/group).

Group	Comedogenic potential[+]	Acanthosis (μm)	Inflammatory reaction
Water control	7.5 ± 0.7^{b}	12.5 ± 1^{b}	Absent
Base	9.0 ± 0.7^{b}	14.5 ± 1^{b}	Absent
Base + BZ-3	13.8 ± 0.5^{a}	26.7 ± 0.9^{a}	Absent
Base + BZ-3-HPCD	9.8 ± 0.5^{b}	16.5 ± 0.9^{b}	Absent

[+] Comedogenic potential: value < 10 (none), 10 < value ⩽ 30 (mild), 30 < value ⩽ 90 (moderate), value > 90 (severe). Values followed by different letters in the same column indicate statistically significant differences ($p < 0.05$).

3.3. Macroscopic and histological evaluation of topical sunscreen formulations

Comedogenicity is an important consideration in the development of topical medications, cosmetics, and skin care products. The term "acne cosmetica" was developed to link the use of certain substances to comedones formation, and animal models have been used to determine the comedogenic potential of finished formulations containing these substances [49].

Macroscopic observations made daily in the ears of rabbits suggested that there were no visible comedones and no irritative signs, such as erythema, epithelial desquamation, and inflammatory reactions, in any group treated with the base, base with BZ-3, and base with BZ-3-HPCD complex in the stoichiometric ratio of 1:2.

The results of a histological analysis are shown in Fig. 8 and Table 1. Statistical analysis indicated a significant difference in the comedogenic potential only for the group treated with the formulation containing BZ-3 (Fig. 8A), which was associated with medium comedones and potential comedones with values ranging from 10 to 30. In the other treatment groups, we observed only small comedones and potential comedones with values below 10 (Fig. 8B). Therefore, it was possible to conclude that the formulation with BZ-3-HPCD complex showed no comedogenic potential.

Concerning the presence of acanthosis, the group treated with the formulation containing only the BZ-3 was also significantly different in that regard at 5%. This group presented mean numbers of comedones and acanthosis sizes that were 29% and 38% higher, respectively, than the group treated with the formulation containing the BZ-3-HPCD complex. Concerning the analysis of inflammatory infiltrates, none of the groups had a positive result (Table 1). The histological findings of this study are consistent with those carried out by Truite et al. [32]. Their formulation of a phytotherapic agent for vitiligo therapeutic propagated through the skin up to the melanocytes region, inducing cutaneous reactions and an increase of the comedogenic effect. Similar studies with the formulation containing the BZ-3-HPCD complex were not found in the literature.

4. Conclusions

Thermal analysis and PAS suggested the complexation of BZ-3 with HPCD in the stoichiometric ratio of 1:2. Histological analysis showed no tissue reaction when employing formulations with the complex. PAS also showed low penetration of sunscreens, which is consistent with results obtained with the use of PAS. Therefore, the use of the BZ-3-HPCD complex in sunscreen formulations is favorable. Moreover, PAS is a methodology that can be used with more conventional approaches to study the formation of inclusion complexes with CDs and to evaluate the percutaneous penetration of the complexes. With these results, it is possible to infer that the formulation with the complex BZ-3-HPCD is a promising candidate to improve the effect of sunscreen formulations and that PAS may be a useful tool to evaluate the photostability of these formulations.

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