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Article

Determination of *In Vitro* Usnic Acid Delivery into Porcine Skin Using a HPLC Method

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Usnic acid, a lichen metabolite, has been proposed as a potential topical treatment for microbial skin lesions, burn wounds as well as a sunscreen. An isocratic HPLC method was validated according to FDA's Guidance for Industry: Bioanalytical Method Validation to determine skin penetration and permeation of usnic acid. The penetration and permeation of usnic acid was evaluated using Franz cells and porcine skin. The method was valid according to selectivity, linearity, precision, accuracy and stability. Usnic acid was quantified in the skin surface (6.13 μg cm^2), *stratum corneum* (34.4 μg cm^2), viable epidermis (5.6 μg cm^2), dermis (28.2 μg cm^2) and receptor compartment (3.2 μg cm^2). These results help us to understand the penetration profile of usnic acid and plan topical therapeutic approaches as well as new topical delivery systems to modulate this penetration profile.

Introduction

Lichens are formed through symbiosis between a fungal and a photosynthetic partner such as algae or cyanobacteria. More than 17,000 species and over 800 lichen products are known. Components such as usnic acid (Figure 1) are utilized for perfumery and for medicinal purposes (1).

Since its first isolation in 1844, usnic acid (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzo-furandione) has become the most extensively studied lichen metabolite and one of the few that is commercially available (2). In humans, it is quite useful in improving burn healing and it can act as an anti-inflammatory, antimitotic, antineoplastic, antibacterial and antimycotic agent (3–6) and displays variable redox-active properties, acting as an antioxidant and pro-oxidant agent, according to different system conditions and/or cellular environment (7).

Besides topical application for burn wounds, usnic acid has been proposed as a potential topical treatment of nosocomially acquired *Staphylococcus aureus* isolates as well as a sunscreen (8). Even though several research studies indicate that the topical application of usnic acid is promising for various treatments, no data on usnic acid permeation through the skin or into the skin are currently available. Even though a chromatographic method has been used for the determination of usnic acid in a biological matrix, the human plasma (9), no studies have focused on the quantification of usnic acid using the skin as a biological matrix.

In order to determine the amount of usnic acid in plasma, the mobile phase required a salt-based buffer, phosphate buffer, which in long-term use can result in precipitation and equipment damage. Noting that the skin is a differentiated biological matrix, it was possible to adjust mobile phase pH with acetic acid. Furthermore, the earlier report suggests the use of an internal standard, diclofenac sodium, which was not necessary in our method (9).

Another research quantified usnic acid in lichens extracts, also using the external standard method and acetic acid as a mobile phase pH adjuster (10). Even though natural product extracts are complex samples, bioanalytical analyses require a differentiated method for recuperating the active substance after application or administration in a biological system.

The purpose of this study was to develop and validate a chromatographic method to quantify usnic acid in the skin and perform a preliminary permeation and penetration study of this substance in porcine skin.

Experimental

Reagents

All solvents were of HPLC grade. Acetonitrile and methanol were from Tedia (Rio de Janeiro, Brazil). Acetic acid and ether (analytical grade) were purchased from Labsynth (Diadema, Brazil). The lauryl sodium sulfate and hydroxyethyl cellulose were from Cosmetrade (Porto Alegre, Brazil). Ethanol, analytical grade, was obtained from Nuclear (São Paulo, Brazil). Usnic acid was acquired from Sigma-Aldrich (Steinheim, Germany). The water used in all experiments was ultrapure.

HPLC conditions

The HPLC equipment consisted in a high-pressure pump, a UV detector and an auto sampler, Series 200 (Perkin Elmer do Brasil Ltda, São Paulo, Brazil) and Total Chrom Software®. The column used was a Luna C-18 5 μm (150 mm × 4.6 mm) (Phenomenex®, Torrance, USA) and guard column C-18 (Phenomenex®, Torrance, USA). The mobile phase used was methanol–water–acetic acid (80:15:5) with a flow rate of 1.0 mL min^{-1}. The detection wavelength was 350 nm and the injection volume was 20 μL.

Method validation

The method was validated according to FDA's Guidance for Industry: Bioanalytical Method Validation (2001) (11). Selectivity was determined using six blank samples of porcine skin and of receptor medium. The lower limit of quantification was accepted to be the lowest standard concentration on the standard curve,

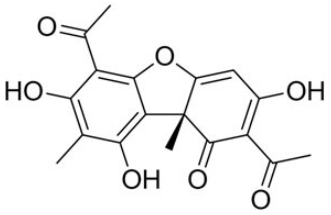

Figure 1. Usnic acid molecular structure.

which had a response over five times the response obtained in the blank samples.

The standard curve was obtained by spiking 2 g of porcine skin cut into small pieces with known concentrations of the usnic acid. The usnic acid concentrations were obtained by varying the volume of a standard solution in acetonitrile (0.5 mg mL^{-1}) and adjusting the final volume to 10 mL, also with acetonitrile, before the extraction procedures. The standard curve was composed of seven non-zero samples (1, 2.5, 5, 10, 15, 20 and 25 μg mL^{-1}). The equation that determines the relationship between concentration and response (area) was the mean of three replicates.

Separately, five replicates of the above-explained procedure at the concentrations 6.5 μg mL^{-1} (low), 15 μg mL^{-1} (medium) and 25 μg mL^{-1} (high) were prepared. The mean and coefficient of variation of the concentration obtained using the standard curve equation were calculated to obtain the accuracy and precision, respectively.

Of the five proposed stability validations (freeze and thaw, short-term temperature, long-term, stock solution and post-preparative), only three met the experimental necessities of collecting, handling and storing conditions. Short-term temperature stability was evaluated by collecting three aliquots of a high (25 μg mL^{-1}) and low (2.5 μg mL^{-1}) concentration samples from the standard curves and keeping them at room temperature for 24 h. Stock solution stability concentration was measured as soon as it was prepared and 24 h after. Post-preparative stability was evaluated by running a standard curve batch twice, with an interval of 24 h.

Gel preparation

To perform topical application of usnic acid, a hydroxyethyl cellulose gel was prepared. Usnic acid (2%) was dispersed in ethanol (16%), and the volume was completed with water, subsequently 2% of hydroxyethyl cellulose was added to the dispersion. The formulation was preserved for 24 h and homogenized manually before use.

Skin preparation

Porcine flank skin was donated from a regional slaughterhouse (Bento Gonçalves, Brazil). Excess fat tissue, hypodermis and hairs were removed, maintaining the full-thickness skin. The skin was clean on the surface with a lauryl sodium sulfate 0.1% solution and on the dermis with an alcohol–ether (1:1) solution before storage at −20°C for a maximum of 3 months. Before each experiment, the skin was cut into round slices, and all skin slices used had width between 1.8 and 2.2 mm.

Skin permeation and penetration assays

The penetration assay was performed with an automatic Franz-type diffusion cell (MicroettePlus Multi-Group®; Hanson Research Corporation, Chatsworth, USA) during 12 h. The receptor medium used to the guarantee sink condition was 7 mL of an aqueous solution of DMSO 2% (v/v). The gel (50 mg) was manually applied on the diffusion area (1.77 cm^2). The cells were occluded with a glass cover. The acceptor phase was maintained under constant stirring and a temperature of 35°C during the whole experiment. Samples of 1 mL were withdrawn from the acceptor phase automatically into vials in determined intervals up to 12 h and these were injected in the HPLC. The collected aliquots were replaced with fresh solution.

At the end of 12 h, the excess gel on the surface of the skin was removed with one tape strip. The *stratum corneum* was obtained by the tape stripping technique, using 18 tape stripes (3M® Brasil; Sumaré, Brazil). Epidermis and dermis were separated using a warm (60°C) water bath for 45 s followed by removal with a spatula. Usnic acid was extracted with acetonitrile from all layers of the skin: skin surface (2 mL), *stratum corneum* (3 mL), *epidermis* (3 mL) and *dermis* (3 mL). The extraction was performed by 2 min of vortex followed by 30 min of sonication. The samples were filtered into vials (0.45 μm, Merck Millipore, Darmstadt, Germany) before HPLC analysis. All results are expressed as mean ± standard deviation ($n = 6$).

Results

Method validation

Usnic acid retention time in this method was 4.3 min (Figure 2), an adequate retention time for drug penetration and permeation assays. The method was selective, no response was observed for blank skins at 4.3 min, the retention time of usnic acid. The standard curve was linear ($r = 0.9994$) and the deviation among the responses (areas) obtained by HPLC was under 20% for the concentration of 1.0 μg mL^{-1} and lower than 15% for all other concentrations. The simplest model that adequately describes the concentration–response relationship was the linear equation: $y = 119\,550.7\,x + 31\,266.7$.

Five replicates with the concentrations 6.5 μg mL^{-1} (low), 15 μg mL^{-1} (medium) and 25 μg mL^{-1} (high) were prepared. The mean response values deviated within 14.9, 4.6 and 1.4%, respectively, indicating that the method is accurate and the coefficient of variation presented values of 2.7, 2.0 and 1.8%, respectively, indicating that the method is precise.

When evaluating the short-term temperature stability of a high (25 μg mL^{-1}), medium (10 μg mL^{-1}) and low (2.5 μg mL^{-1}) concentration samples at room temperature for 24 h, concentration decrease was observed (2.9, 4.8 and 5.7%, respectively). Stock solution stability concentration was measured as soon as it was prepared and 24 h after and no concentration decrease was observed. When submitted to the post-preparative stability assay, the samples exhibited that there was no variation of concentration.

Skin permeation and penetration assay

During the period of the assay, it was only possible to quantify usnic acid in the receptor medium at the end of 12 h. The penetration profile is presented in Figure 3. Usnic acid was capable of

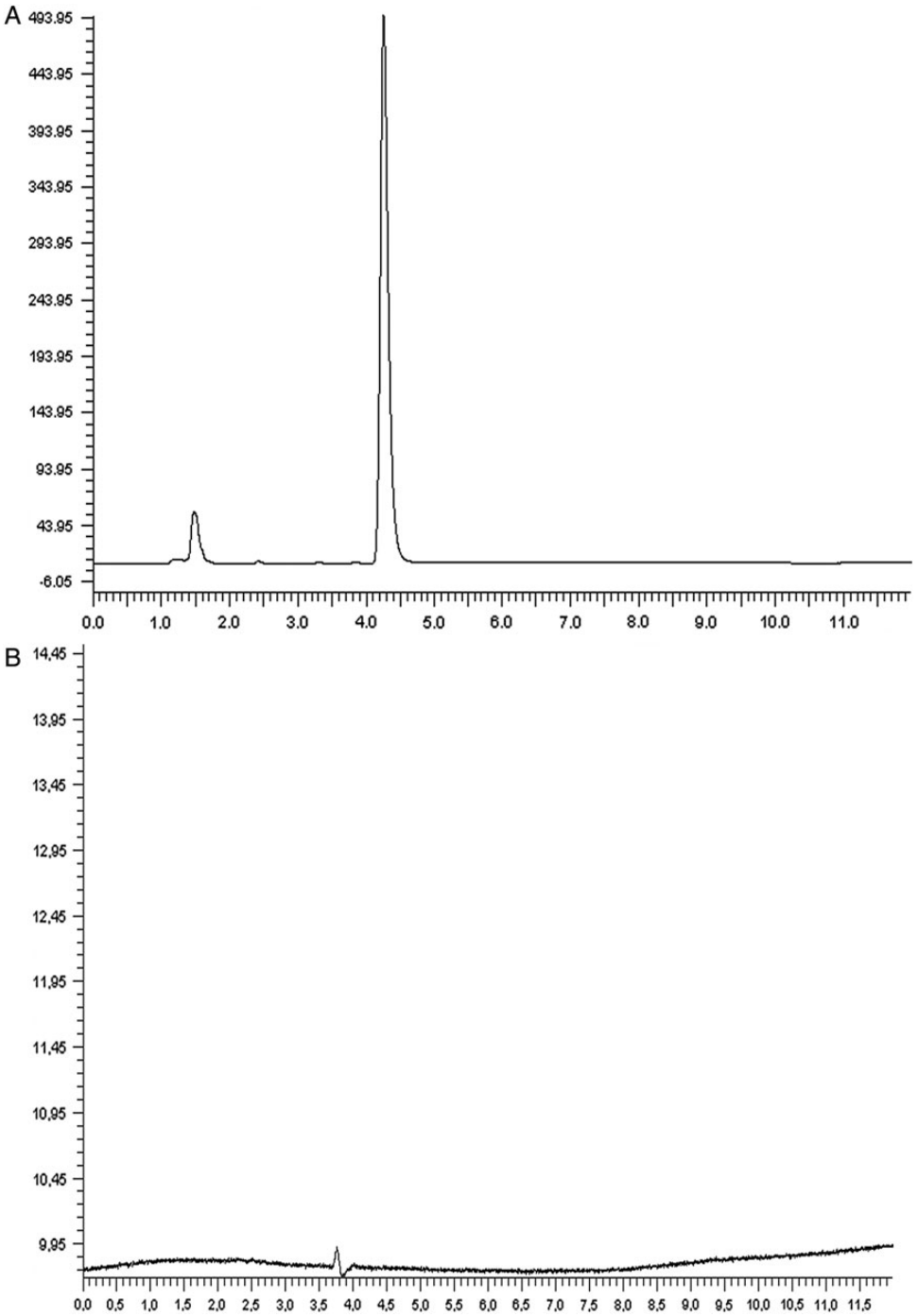

Figure 2. HPLC chromatogram of usnic acid (A) and blank porcine skin (B), using mobile phase of methanol–water–acetic acid (80:15:5) with a flow rate of 1.0 mL min^{-1} and detection wavelength of 350 nm.

penetrating the skin and was quantified in the *stratum corneum*, viable epidermis and dermis. After 12 h, a small amount of usnic acid was still found on the surface of the skin (6.13 μg cm^{-2}). Usnic acid penetrated greatly into the *stratum corneum* (34.4 μg cm^{-2}). The viable epidermis accumulated a small amount (5.6 μg cm^{-2}) of the substance. A larger amount of usnic acid was found in the dermis (28.2 μg cm^{-2}). After 12 h the transdermal permeation of usnic acid was very small (3.2 μg cm^{-2}).

Discussion

Analytical methods, employed for the quantitative determination of drugs in biological samples, can influence the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data. It is therefore essential to employ well-characterized and fully validated analytical methods to give reliable results and which can be interpreted (12, 13). However, as far as we know, no quantitative method for the determination of usnic acid in skin has been reported in the literature. Therefore, a new

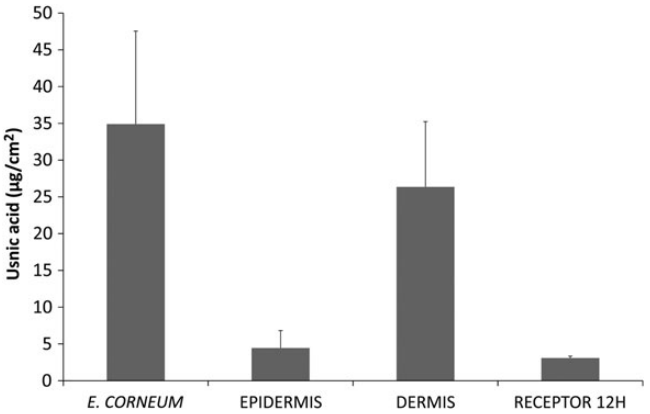

Figure 3. Skin penetration profile of usnic acid dispersed in hydroxyethyl cellulose gel.

method for the quantification of usnic acid in the skin by liquid chromatography was developed for a preliminary study on the transdermal penetration of usnic acid after its topical application.

The HPLC method for a rapid, precise and accurate determination of transdermal permeation and cutaneous penetration of usnic acid have been developed and validated in this study.

The stability assays suggest that the penetration/permeation experiment should not be interrupted or left overnight due to a possible concentration decrease, even if small. The proposed method shows a promising applicability in determining usnic acid penetration and permeation from different cutaneous pharmaceutical forms. Penetration/permeation assays are very important in the research and development of cutaneous pharmaceutical forms and usnic acid is precisely in the research and development phase.

The *stratum corneum* most probably acted as a reservoir system for usnic acid that penetrated greatly (34.4 $\mu g\ cm^{-2}$) into this layer of the skin. Viable epidermis did not act as a barrier for the molecule as seen by the small amount (5.6 $\mu g\ cm^2$) of the substance found in this layer. Usnic acid also tended to accumulate in the dermis (28.2 $\mu g\ cm^2$) and would probably lead to a systemic absorption. After 12 h the transdermal permeation of usnic acid was very small (3.2 $\mu g\ cm^2$), but considering that the formulation used was very simple and did not intend to favor this route, the use of a transdermal application seems viable.

The *in vitro* experiment was unable to determine the permeation coefficient and flux due to a low permeation of the drug. Still, it is possible to mathematically predict these parameters based on the molecule properties. Usnic acid physicochemical characteristics are suitable for cutaneous application, considering that, in general, molecules should have a molecular weight lower than 500 and an octanol/water partition coefficient (K_{ow}) between 10 and 100 in order to penetrate the skin. Usnic acid molecular weight is 344.3 and $K_{ow} = 75.9$ ($\log P = 1.88$) rendering a permeation coefficient (K_p) of 3.12×10^{-4} cm h^{-1}, using the Potts and Guy method.

The theoretical calculation of the permeation coefficient in this case represents an overestimation of the experimentally observed penetration; a similar observation was made by Mestres and coworkers (2011) (14). The penetration and permeation experiment favor a topical delivery of usnic acid instead of a transdermal delivery.

Conclusion

It was possible to develop and validate a simple, isocratic HPLC method useful in determining skin penetration and permeation of usnic acid. The method was used to determine the accumulated amount of usnic acid in the different layers of the skin as well as the amount that permeated *in vitro*. These results help to understand the penetration profile of usnic acid and plan topical therapeutic approaches as well as plan new topical delivery systems to modulate this penetration profile.

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