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Evaluation of critical parameters for *in vitro* skin permeation and penetration studies using animal skin models

Fabíola Silva Garcia Praça[a,*], Wanessa Silva Garcia Medina[b], Josimar O. Eloy[a,1], Raquel Petrilli[a], Patrícia Mazureki Campos[a], Andreia Ascenso[c], Maria Vitória L.B. Bentley[a]

[a] *School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Avenida do Café, s/n, 14040903 Ribeirão Preto, SP, Brazil*
[b] *Faculdades Integradas Padre Albino – FIPA, Catanduva, SP, Brazil*
[c] *Research Institute for Medicines (iMed.ULisboa), Faculdade de Farmácia, University of Lisbon, Portugal*

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ABSTRACT

In vitro skin permeation/penetration studies may be affected by many sources of variation. Herein, we aimed to investigate the major critical procedures of *in vitro* skin delivery studies. These experiments were performed with model drugs according to official guidelines. The influence of skin source on penetration studies was studied as well as the use of a cryopreservation agent on skin freezing evaluated by transepidermal water loss, electrical resistance, permeation/penetration profiles and histological changes of the skin. The best condition for tape stripping procedure was validated through the evaluation of the distribution of corneocytes, mass of stratum corneum (SC) removed and amount of protein removed using finger pressure, a 2 kg weight and a roller. The interchangeability of the tape stripping procedures followed by the epidermis and dermis homogenate and the micrometric horizontal cryostat skin sectioning methods were also investigated, besides the effect of different formulations. Noteworthy, different skin sources were able to ensure reliable interchangeability for *in vitro* permeation studies. Furthermore, an increased penetration was obtained for stored frozen skin compared to fresh skin, even with the addition of a cryoprotectant agent. The best method for tape stripping was the finger pressure followed by the addition of a propylene glycol solvent leading to better SC removal. Finally, no significant difference was found in skin penetration studies performed by different methods suggesting their possible interchangeability.

1. Introduction

The skin is considered an important route for both topical and systemic administration of drugs. Ideally, topical delivery should be previously tested *in vivo* in order to study the delivery system, toxicity and irritation potential of unknown drugs, among other parameters (Haigh and Smith, 1994). Despite excised human skin is considered the most appropriate model (gold standard) for *in vitro* permeation and penetration experiments, some disadvantages lead to use other models. Major drawbacks include poor convenience, difficult obtainment and low standardization level due to variability related to gender, race, age and anatomical site of the donor (Barbero and Frasch, 2009). Thus, herein the use of human skin was avoided and the purpose of this study was to provide a comparison of various animal skin types that could serve as skin membrane models for *in-vitro* penetration studies. The use of porcine ear skin, hairless mouse skin and shed snake skin has been widely studied as animal model membranes to replace human skin (Vecchia and Bunge, 2005; Itoh et al., 1990a; Itoh et al., 1990b; Rigg and Barry, 1990) as well as to evaluate skin permeation enhancers (Campos et al., 2016; Petrilli et al., 2013; Praça et al., 2012; Baby et al., 2008; Lopes et al., 2007; Nunes et al., 2005; Nicolazzo et al., 2003; Hirvonen et al., 1991), once they are easily available, easy to employ and can provide results rapidly (Jung and Maibach, 2015). Porcine ear skin and hairless mouse skin are widely employed, while shed snake skin has also potential to be used as a membrane model based on stratum corneum (SC) penetration rate (Praça et al., 2012; Baby et al., 2008; Nunes et al., 2005). On the other hand some drawbacks of these alternative membranes should be considered. Porcine ear skin has similar histological characteristics compared to human skin. It presents similar SC thickness and hair-follicle density. However, SC composition

* Corresponding author.
E-mail addresses: fgarcia@fcfrp.usp.br (F.S.G. Praça), wasigame@gmail.com (W.S.G. Medina), josimar.eloy@gmail.com (J.O. Eloy), raquelpetrilli@yahoo.com.br (R. Petrilli), patimazureki@hotmail.com (P.M. Campos), andreiaascenso@ff.ul.pt (A. Ascenso), vbentley@usp.br (M.V.L.B. Bentley).
[1] Present address: School of Pharmaceutical Sciences, UNESP – São Paulo State University, Campus Araraquara, Department of Drugs and Medicines, Rodovia Araraquara-Jau, km. 1, Araraquara, São Paulo 14,801–902, Brazil.

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of both species differs, because porcine SC presents the lipids organized in hexagonal lattice, whereas human SC is in orthorhombic lattice. Furthermore, in order to obtain this kind of skin it is necessary to have animal slaughterhouse facilities nearby that offer skin in adequate conditions for the experiments. Other hairless mouse species are used and because of the absence of hair, they have similarities with human skin. This model is advantageous because of their small size, low cost and easy handling (Depieri et al., 2015) but one of the major drawbacks is related to increased drug permeation rates compared with human skin, and need for approval of the ethics committee. In the case of snake skin, the samples are obtained without the sacrifice of the animals, are easy to store and do not suffer microbiological deterioration, besides, this model membrane contemplates the aspect of experimental animal ethics and is ecologically correct (Baby et al., 2006). However, this skin is not easy to obtain in a conventional lab and the absence of hair follicles is a disadvantage that limits its use (Godin and Touitou, 2007). Although some studies have already used shed snake skin as a biological membrane for *in vitro* permeation tests (Baby et al., 2008), there is still a lack of comparative studies that ensure the interchangeability with other animal skins. Thus, the use of porcine ear skin and hairless mouse skin remain the general choice (World Health Organization, 2006).

Furthermore, skin storage is usually required for both *in vitro* drug permeation and topical drug recovery studies, and consequently, it is quite important that each laboratory defines the most suitable storage conditions (Barbero and Frasch, 2009). Curiously, some authors reported that epithelial membrane previously frozen did not change the permeation profiles of different drugs (Nicolazzo et al., 2003). Conversely, both the permeability and the lag time of hydrophobic drugs were significantly affected by skin freezing (Barbero and Frasch, 2009; Ahlstrom et al., 2007). The use of a cryoprotectant agent such as 10% glycerol was previously suggested (Liangpeng et al., 2011; Bravo et al., 2000; Richters et al., 1996) in order to avoid ice crystal formation and maintain the skin barrier function. However, there is no consensus yet about the use of a skin cryoprotectant agent and the storage time of frozen skin used for *in vitro* permeation/penetration studies (Petrilli et al., 2016; Estracanholli et al., 2014; Liangpeng et al., 2011; Barbero and Frasch, 2009).

Different protocols are available to quantify the drug retained into porcine or hairless mouse skins, such as (i) evaluation of drug concentration in SC by the tape stripping technique (Lademann et al., 2009), (ii) evaluation of drug concentration in deeper skin layers by homogenization of skin tissue after tape stripping (viable epidermis without SC and dermis) (Campos et al., 2016; Depieri et al., 2015; Ascenso et al., 2014; Praça et al., 2011), (iii) evaluation of drug concentration in the whole skin tissue by homogenization (Ascenso et al., 2013), and (iv) assessment of skin drug penetration by specific micrometric sectioning of horizontal skin using a cryostat (Praça et al., 2012; Labouta et al., 2011; Echevarria et al., 2003). Among these methods, the homogenization technique of the whole skin tissue is easier, faster and less laborious, being also quite useful to detect a low drug penetration level. Nevertheless, it does not provide data on specific drug skin localization. Tape stripping appears to be simple and easy to perform being useful to assess the distribution and amount of drug in the SC. However, the number of strips and the applied pressure are parameters that should be clearly defined since they may influence the outcome. Some researchers related the uniform pressure on the skin with the amount of SC removed. Protein in corneocytes is the major component of the SC, and this evaluation has been considered a standard technique to quantify the removed SC amount. Meanwhile, the quantification of the penetrated drug in each strip or in the aggregate strips depends on sensitivity of the analytical method. Therefore, most studies quantify the penetrated drug into SC using the aggregate of all tape strips (Campos et al., 2016; Estracanholli et al., 2014; Praça et al., 2011; Rossetti et al., 2010; Herkenne et al., 2006).

Basic knowledge of these underlying critical parameters for skin permeation/penetration studies is an essential aspect for achieving a successful research. An extensive database has been generated over the past years due to numerous *in vitro* experiments aimed to improve the delivery of topically applied drugs and cosmetics. Nonetheless, few data on the experimental reproducibility based on skin source, skin storage conditions and skin drug recovery rate is available (Praça et al., 2011; Barbero and Frasch, 2009; Lademann et al., 2009; Baby et al., 2008; Ahlstrom et al., 2007; Ngawhirunpat et al., 2006; Baby et al., 2006; Nunes et al., 2005; Löffler et al., 2004; Babu et al., 2003; Takahashi et al., 2001; Pongjanyakul et al., 2000; Haigh et al., 1998; Fang et al., 1995; Hirvonen et al., 1991; Itoh et al., 1990b; Rigg and Barry, 1990; Bronaugh and Stewart, 1985) and none of the studies summarized all these parameters in a single report, which has been addressed herein. Therefore, the main purpose of the present work was to investigate and standardize critical parameters for *in vitro* skin permeation/penetration studies using animal skin models and thus minimize experimental errors. To our knowledge, this is the first report in which these parameters are correlated.

In order to standardize the protocols for *in vitro* skin penetration, nicotine, estradiol and fluorescein were used as model drugs and fluorescent probe, respectively. In particular, the specific aims of this work were: (a) to observe the influence of animal skin source on penetration studies; (b) to evaluate the influence of a cryoprotectant agent on skin freezing by transepidermal water loss, electrical resistance, penetration profiles and skin histological changes; (c) to explore the critical parameters involved in drug quantification in different skin layers, such as: the influence of uniform pressure applied in the tape stripping process; the effect of different vehicles composition on tape stripping procedure after *in vitro* skin permeation, and the interchangeability between the conventional tape stripping followed by epidermis and dermis (EP + D) homogenization and the micrometric horizontal cryostat skin sectioning for the evaluation of skin drug penetration; (d) to evaluate different conditions for nicotine recovery from skin sample.

2. Materials and methods

2.1. Materials

Nicotine, estradiol and fluorescein (6-carboxylfluorescein) used to prepare standards for HPLC and spectrometric determinations were purchased from Sigma (St. Louis, MO, U.S.A.). Transdermal delivery systems used in this study were Niquitin™ 5.1 mg/cm^2 (each 15 cm^2 contains 78 mg of nicotine) from GlaxoSmithKline (Rio de Janeiro, Brazil) and Estradot® 0.156 mg/cm^2 (each 10 cm^2 contains 1.56 mg of estradiol) from Novartis (São Paulo, Brazil). Aqueous solutions were prepared using ultrapure water obtained from a Water Purification System (Millipore Corporation, MA, U.S.A.) and all other reagents or solvents were of analytical or high performance liquid chromatography (HPLC) grade.

2.2. Methods

2.2.1. Comparison of different skin sources on in vitro drug permeation assay

2.2.1.1. Hairless mouse skin. 6 week old male hairless mouse (HRS/J strain) weighing 20–30 g were obtained from the colony of the School of Pharmaceutical Sciences of Ribeirão Preto (University of São Paulo, Ribeirão Preto, SP, Brazil). They were housed in a temperature and humidity-controlled room, with access to food and water *ad libitum*. The procedures were approved by the Ethics Committee of the School of Pharmaceutical Science of Ribeirão Preto (University of São Paulo, Ribeirão Preto, SP, Brazil – Process #09.1.505.53.6).

2.2.1.2. Porcine ear skin. Porcine ear skin samples obtained from a local slaughterhouse were carefully dissected (ensuring that the

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