European Journal of Pharmaceutical Sciences 60 (2014) 49–54

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

An improved cryopreservation method for porcine buccal mucosa in *ex vivo* drug permeation studies using Franz diffusion cells

Sonia Amores [a], José Domenech [a], Helena Colom [a], Ana C. Calpena [a], Beatriz Clares [b,*], Álvaro Gimeno [c], Jacinto Lauroba [a]

[a] Department of Biopharmacy and Pharmaceutical Technology, School of Pharmacy, University of Barcelona, Barcelona, Spain
[b] Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Granada, Granada, Spain
[c] Animal Facility, Bellvitge Health Sciences Campus, University of Barcelona, Barcelona, Spain

ARTICLE INFO

Article history:
Received 25 December 2013
Received in revised form 21 April 2014
Accepted 26 April 2014
Available online 9 May 2014

Keywords:
Buccal drug delivery
In vitro models
Cryopreservation
Permeability
Diffusion
Transmucosal water loss

ABSTRACT

The use of isolated animal models to assess percutaneous absorption of molecules is frequently reported. The porcine buccal mucosa has been proposed as a substitute for the buccal mucosa barrier on *ex vivo* permeability studies avoiding unnecessary sacrifice of animals. But it is not always easy to obtain fresh buccal mucosa. Consequently, human and porcine buccal mucosa is sometimes frozen and stored in liquid nitrogen, but this procedure is not always feasible. One cheaper and simpler alternative is to freeze the buccal mucosa of freshly slaughtered pigs in a mechanical freezer, using DMSO and albumin as cryoprotective agents. This study compared the *ex vivo* permeability parameters of propranolol hydrochloride through porcine buccal mucosa using a Franz diffusion cell system and HPLC as detection method. The freezing effects on drug permeability parameters were evaluated. Equally histological studies were performed. Furthermore, the use of the parameter transmucosal water loss (TMWL) as an indicator of the buccal mucosa integrity was evaluated just as transepidermal water loss (TEWL) is utilized for skin integrity. The results showed no difference between fresh and frozen mucosal flux, permeability coefficient or lag time of propranolol. However, statistical significant difference in TMWL between fresh and frozen mucosa was observed.

1. Introduction

In recent years, most biopharmaceutical and pharmacokinetic research has focused either on the use of new routes for drug administration or on new drug delivery systems, with the aim of obtaining improved therapeutic activity, fewer adverse effects or better patient compliance (Holm et al., 2013).

Owing to the ease of the administration, the oral cavity is an attractive site for the delivery of drugs. Through this route it is possible to realize mucosal (local effect) and transmucosal (systemic effect) drug administration (Schwarz et al., 2013). The buccal mucosa appears to be better in terms of permeability, surface area, compliance, etc., when compared to the other mucosal and transdermal routes of delivery (Kulkarni et al., 2010).

Drug delivery in the oral cavity is a logical alternative delivery route for drugs which undergo extensive degradation in the stomach and the liver. Three types of oral mucosa can be found in the oral cavity. The lining mucosa (60%), the masticatory mucosa (25%) and the specialized mucosa (15%), all these represent an available surface of 170 cm^2 for drug absorption, of which ~50 cm^2 represents non-keratinized tissues (Patel et al., 2011). The permeability of buccal mucosa is between 4 and 4000 times greater than that of skin. As a result, faster onset of action for several drugs has been observed (Galey et al., 1976).

Permeation experiments are a valuable adjunct to *in vivo* percutaneous absorption studies, and provide a convenient means for evaluating the permeation characteristics of drugs (Bronaugh and Maibach, 1991). A variety of passive diffusion systems for *in vitro* permeation experiments have been developed for use with different kinds of membranes. For *in vitro* transdermal studies, Franz diffusion cells are perhaps the most commonly used setups. Human buccal mucosa is scarcely available, thus most research efforts relied on the use of isolated animal buccal tissue. *In vitro* and *ex vivo* methods have been helpful for preclinical drug screening as well as elucidating mechanisms of transport across the buccal mucosa, or even evaluation of potential chemical penetration enhancers (Nicolazzo and Finnin, 2008).

* Corresponding author. Address: Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Granada, Campus de la Cartuja s/n, 18071 Granada, Spain. Tel.: +34 958 243904; fax: +34 958 248958.
E-mail address: beatrizclares@ugr.es (B. Clares).

http://dx.doi.org/10.1016/j.ejps.2014.04.017
0928-0987/

When compared to the other animal models, porcine buccal mucosa has been considered the most representative model for human tissue due to its close resemblance to human buccal mucosa in ultra-structure (non-keratinised) and enzyme activity (Diaz-del Consuelo et al., 2005; Fernández-Campos et al., 2012).

Since it is not always practical to perform permeability experiments within hours of receiving tissue, once obtained the porcine buccal mucosa, conditions of storage play an important role, it is necessary to be able to bank buccal mucosa. The most significant question concerning the use of animal tissue in such a manner is the viability and integrity of the dissected tissue (Patel et al., 2011).

One method for achieving this has been to collect human and porcine buccal mucosa in liquid nitrogen and to store it for later use (Veuillez et al., 2002; Van Eyk and Van der Bijl, 2006). However, the liquid nitrogen procedure is not always feasible. Other storage conditions such as phosphate buffer saline (PBS) pH 7.4 (4 °C), dry wrapped in aluminium (−20 °C) or cryoprotected in 20% glycerol solution (−20 °C) for either 6, 24 or 48 h resulted in loss of epithelial integrity (Patel et al., 2011).

An alternative method consisting of PBS mixture containing 4% albumin and 10% DMSO, which has been used to date for freezing of living cells (Galmes et al., 2007; Rowley et al., 1994) was evaluated in the current study. Porcine buccal mucosa was placed in containers with cryoprotective agents, and frozen in the same mechanical freezer. This procedure is both cheaper and simpler, and it avoids the possible contamination associated with the use of nitrogen tanks (Tedder et al., 1995).

Fluorescein isothiocyanate has been established as an integrity marker for porcine buccal mucosa, whilst transepidermal water loss (TEWL) has been used to monitor skin integrity (Netzlaff et al., 2006; Sierra et al., 2013). TEWL has also been used to measure water loss in human nasal mucosa (Miwa et al., 2006). The possibility of using transmucosal water loss (TMWL) to monitor porcine buccal mucosa integrity was investigated. Propranolol (PP) was used as a drug model for buccal delivery because (i) is a potent drug, (ii) medium apparent aqueous solubility (Yang and Fassihi,1997), (iii) first past metabolism after oral administration (Lalka et al., 1993) and (iv) suitable for pH-dependent absorption studies as PP is a secondary amine, with a pK_a value around 9.53 (Wishart et al., 2008).

The aim of the current research was to study PP permeability and porcine buccal mucosa integrity by comparing the following biopharmaceutical parameters: flux, permeability coefficient, lag time and TMWL using fresh or frozen porcine buccal mucosa from the same subject.

2. Materials and methods

2.1. Chemicals

Propranolol hydrochloride was obtained from Acofarma (Terrassa, Spain). Hank's balanced salt solution (HBSS) (Composition in g/L: $CaCl_2$ = 0.14; KCl = 0.14; KH_2PO_4 = 0.06; $MgSO_4$ = 0.1; $MgCl_2$ = 0.1; NaCl = 8.0; $NaHCO_3$ = 0.35; Na_2HPO_4 = 0.09; Glucose = 1) was obtained from Biological Industries (Kibbutz Beit Haemek, Israel). PBS was obtained from Sigma–Aldrich (Madrid, Spain). Albumin solution 4% was obtained from Laboratorios Grifols (Barcelona, Spain). Dimethyl sulfoxide (DMSO) was supplied by Merck Lab. (Madrid, Spain). Acetonitrile, acetic acid, sodium phosphate and potassium phosphate were purchased from Panreac Química (Barcelona, Spain). All chemicals were analytical grade and were used without further purification.

2.2. Preparation of the porcine buccal mucosa

The studies were conducted under a protocol approved by the Animal Experimentation Ethics Committee of the University of Barcelona (Spain) and the Committee of Animal Experimentation of the regional autonomous government of Catalonia (Spain). 3–4-month-old female pigs were used (n = 15). The porcine buccal mucosa was obtained immediately after the pigs had been slaughtered in the Animal Facility at Bellvitge Campus (University of Barcelona, Spain). The animals were slaughtered using an overdose of sodium thiopental anaesthesia. For these studies, both fresh and frozen porcine buccal mucosa utilized came from the same subject, to minimize variability. In this study, 27 replications for the fresh and 22 replications for the frozen buccal mucosa of 15 pigs were carried out.

The fresh buccal tissues were transferred from the hospital to the laboratory in containers filled with Hank's liquid. The other buccal tissues were frozen at the Animal Facility by placing them in containers with a PBS mixture containing 4% albumin and 10% DMSO (as cryoprotective agents) and stored (for a maximum of 1 month) at −80 °C in a mechanical freezer. These buccal specimens were subsequently placed in containers with Hank's liquid and transferred from the Animal Facility to the laboratory. DMSO produces adverse effects at room temperature; therefore, the addition of DMSO prior to freezing was performed at 4 °C, whilst thawing involved immersion in a water bath filled with PBS at 37 ± 1 °C and gentle shaking for 30 min, until total elimination of DMSO was achieved (Rowley et al., 1994).

2.3. TMWL measurement

Prior to placing the solution in question in the Franz cell donor compartment, TMWL (expressed in grams per square meter and hour) was measured *in vitro* to confirm the physical integrity of the buccal mucosa. Before collecting the porcine buccal mucosa, TMWL was measured *in vivo* in recently anesthetized pigs (to avoid salivation loss), obtaining a maximum value of 30 g/h m^2. This value was chosen as cut-off point. Each measure was performed in triplicate using a DermaLab® module (*Cortex Technology*, Hadsund, Denmark) by placing the metering device perpendicular to the surface of the tissue and reaching a stable TMWL reading in 60 s approximately. TMWL is defined as the measurement of the quantity of water that passes from inside the body through the epidermal layer of the skin or the outer layer of the mucosa to the surrounding atmosphere via diffusion and evaporation processes (Netzlaff et al., 2006).

2.4. Franz diffusion cell experiments

Different areas of porcine buccal mucosa have different pattern of permeability, there is significantly higher permeability in the region behind the lips in comparison to cheek region, because in porcine buccal mucosa, the epithelium acts as a permeability barrier, and the thickness of the cheek epithelium is greater than that of the region behind the lips (Harris and Robinson, 1992). For the permeation studies, the fresh or frozen porcine buccal mucosa from the same area was cut to 500 ± 50 μm thick sheets, which contributes to the diffusional barrier (Sudhakar et al., 2006), were obtained using an electric dermatome (model GA 630, Aesculap, Tuttlingen, Germany) and trimmed with surgical scissors in adequate pieces. All devices utilized were previously sterilized. The majority of the underlying connective tissue was removed with a scalpel. The tissue handling was done by following basic safety standards for protection against possible exposure to pathogens.

Then membranes were then mounted in specially designed membrane holders with a permeation orifice diameter of 9 mm (diffusion area 0.63 cm^2). Using the membrane holder, each porcine buccal membrane was mounted between the donor (1.5 mL) and the receptor (6 mL) compartments with the epithelium faced the donor chamber and the connective tissue region facing the