Phospholipid Vesicle-Based Permeation Assay and EpiSkin® in Assessment of Drug Therapies Destined for Skin Administration

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ABSTRACT: Cost-effective and efficient methods for permeability screening are crucial during early development of drugs, drug formulations, and cosmeceuticals. Alternatives to animal experiments are impelled for both economical and ethical reasons. The aim of this study was to determine the ability of the phospholipid vesicle-based permeation assay (PVPA) to assess the effect of different formulations on drug permeability and thus establish its utility in formulation development. Three model drugs were tested in solutions and as liposomal formulations. The permeability results for the PVPA models were compared with the results for the reconstructed human skin model, EpiSkin®. The drugs were ranked based on their estimated penetration potentials, and the results were in accordance with what was expected considering the physicochemical properties of the drugs. $PVPA_s$ (E-80, ceramide, cholesterol, cholesteryl sulfate, and palmitic acid) was able to distinguish between drug solutions and liposomal formulations; however, EpiSkin® detected only small differences between the drugs in solution and formulations. In contrast with EpiSkin®, which is limited by a 3-day testing window, PVPA barriers can be stored frozen for up to 2 weeks or even up to 16 months, depending on their compositions. The PVPA models are thus more cost effective and efficient than the EpiSkin® model for permeability screening during early drug development. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:1119–1127, 2015

Keywords: permeability; reconstructed human epidermis; PVPA; EpiSkin®; skin; liposomes; lipids; *in vitro*; transdermal drug delivery

INTRODUCTION

Topical skin drug delivery is attractive, noninvasive, and painless, and usually involves less adverse reactions compared with systemic delivery. In addition, the skin is an easily accessible administration site.[1] The choice of formulation can influence whether a drug will exert local or systemic transdermal effects. In addition to affecting the delivery of a drug, formulations can also affect the epidermis by providing lubrication, hydration, occlusion, and barrier protection, and even causing it to dry out.[2] Liposomes have been extensively studied for decades as carriers for dermal drug delivery and as active carriers in cosmeceuticals. They have been shown to fuse with skin lipids and sometimes dehydrate the skin after topical administration.[3,4]

During topical formulation development, different characteristics of liposomes as drug carriers need to be controlled and different strategies must be applied to either enhance the penetration of compounds through skin or to promote their deposition into the skin for local drug effects.[3,5] Toxicity and pharmacokinetic studies are also important for the optimization of topical formulations. Therefore, during the early phase of formulation development, reliable and cost-effective screening methods are crucial. To optimize the composition of drug carriers/vehicles and drug-in-liposome characteristics, models for permeability screening will help to identify the best candidates for further development. The *stratum corneum* is the main barrier of the skin,[6] and the lipids found in corneocytes and their arrangements are important for the barrier function.[7] Several *in vitro* models have been proposed that mimic the *stratum corneum*, for example, silicon model membranes, although they lack crucial lipids found in the *stratum corneum*.[8,9] The parallel artificial membrane permeation assays (PAMPA) for skin, on the other hand, contain crucial skin lipids but lack cell-like structures.[10] Animal skin in Franz diffusion cells is often used as a model for estimating skin penetration using either full-thickness skin samples or isolated *stratum corneum*. However, difficulties during skin sample preparation and biological variations can generate complications during data interpretation, and a substantial number of replicates is needed to generate reliable data.[11] Although animal models can offer some useful data when assessing topical formulations, their cost, as well as new regulations and a progressing consensus between government/regulatory, research, teaching, industry, and animal welfare organizations to promote the three Rs (replace, reduce, and refine), is limiting their utility.

Recently, the phospholipid vesicle-based permeation assay ($PVPA_s$; E-80, ceramide, cholesterol, cholesteryl sulfate, and palmitic acid), which includes a barrier containing the main lipid classes found in the skin, was introduced as a simple and reproducible model for predicting skin permeability.[12] The PVPA model was originally developed as a robust, high-to-medium throughput permeability screening model for estimating intestinal permeability[13,14] and was later used for both lead compound selection[15] and formulation optimization.[16,17] PVPA barriers consist of liposomes on a filter support and therefore mimic biological cells and membranes. The fact that different lipids can be incorporated into PVPA barriers to closely resemble various biological barriers makes this

Abbreviations used: ACV, acyclovir; ACV-PC, liposomes of PC (S 100) and acyclovir; ACV-PC/PG, liposomes of PC (S 100), EPG-Na and acyclovir; CF, caffeine; CAM, chloramphenicol; CAM-PC, liposomes of PC (S 100) and CAM; PVPA, phospholipid vesicle-based permeation assay; PBS, Dulbecco's phosphate-buffered saline.

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model promising for the assessment of drug administration at various sites. For example, our PVPA models mimicking the *stratum corneum* have also been used as *in vitro* models in the development of (trans)dermal formulations.[2] In the present study, PVPA models were used for estimating the *stratum corneum* penetration of model drugs and their liposomal formulations.

As an alternative, reconstructed human skin models, such as EpiSkin®, Skinethic®, and EpiDerm®, have been proposed for permeability studies as well as for phototoxicity, irritancy, and corrosiveness testing.[18,19] The barrier in the EpiSkin® model consists of a reconstructed epidermis on a collagen support. EpiSkin® kits are prepared in Costar® well plates.[11,20] The protocol is rather complex, and the inclusion of *stratum corneum* cells from human donors makes this model expensive. EpiSkin® is optimized for safety testing,[21] and several applications have been described and recommended by the Organization for Economic Co-operation and Development.[22] Moreover, EpiSkin® can be used for the testing of irritants and skin metabolism as well as skin absorption.[20,21] It has also been used to test various topical formulations and vehicle effects.[11,23,24] Therefore, because of its resemblance to human epidermis and its ability to be used directly in Costar® well plates, the EpiSkin® model was chosen in this study for a comparison with our *in vitro* PVPA *stratum corneum* models.

The aim of this study was to demonstrate the abilities of PVPA models to assess the effects of different drug formulations to establish their utility in drug development. This was done by testing three different model drugs in solutions or as liposomal formulations and comparing the permeability results from the $PVPA_c$ (E-80 and cholesterol) and $PVPA_s$ models with those from the much more complex and expensive reconstructed human skin model, EpiSkin®. Acyclovir (ACV), chloramphenicol (CAM), and caffeine (CF) were chosen as model drugs to cover broad ranges of lipophilicity and molecular size.

MATERIALS AND METHODS

Materials

Acyclovir, CF, calcein, and ceramides from the bovine spinal cord; CAM, cholesterol, cholesteryl sulfate, ethanol, methanol, palmitic acid, Dulbecco's phosphate-buffered saline (PBS), and sodium hydroxide (NaOH) were obtained from Sigma–Aldrich (St. Louis, Missouri). Acetic acid (glacial) and chloroform were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (HCl, 37%, w/w) was purchased from VWR International (Leuven, Belgium). Egg phospholipid Lipoid E-80, soy phosphatidylcholine (PC) Lipoid S 100, and egg phosphatidylglycerol (PG) sodium Lipoid EPG-Na were obtained from Lipoid (Ludwigshafen, Germany). Mixed cellulose ester filters (0.65 μm pore size) and isopore filters (0.8 and 1.2 μm pore sizes) were purchased from Millipore (Billerica, Massachusetts). Filter inserts (Transwell, $d = 6.5$ mm) and plates were purchased from Corning Inc. (New York, New York). Nucleopore filters (0.4 μm pore size) were obtained from Whatman (part of GE Healthcare, Oslo, Norway). EpiSkin® (aged 13 days, large/1.07 cm^2 surface area) was purchased from SkinEthic Laboratories (Lyon, France).

Methods

Preparation of Liposomes Containing ACV or CAM

Liposomes containing ACV or CAM were prepared by the film hydration method. Three different formulations were prepared, two of which contained PC as the only lipid and one of which contained a mixture of PC and PG. PC (200 mg) was dissolved together with either CAM or ACV (20 mg) in chloroform. PC (180 mg), PG (20 mg), and ACV (20 mg) were dissolved in chloroform and MeOH (1:10, v/v). Organic solvents were removed under vacuum, and lipid films were hydrated with distilled water (10 mL) to form the liposomal dispersions liposomes of PC (S 100) and CAM (CAM-PC), liposomes of PC (S 100) and acyclovir (ACV-PC), and liposomes of PC (S 100), EPG-Na and acyclovir (ACV-PC/PG), respectively. Liposomes were stored at 2–8°C for at least 24 h before further use. They were extruded three times through 0.8 μm filters by nitrogen-driven extrusion (Lauda Dr. R. Wobser Gmbh, Königshofen, Germany).

Size Distribution and Zeta Potential Measurements

The size distribution and polydispersity indices (PIs) of liposomes were determined by photon correlation spectroscopy using Particle Sizer 370 (PSS Nicomp Particle Sizing Systems, Santa Barbara, California). The sample preparation and measuring conditions were the same as previously described.[17] The measurements were performed in three cycles of 10 min each. The zeta potential measurements of the liposomal dispersions ACV-PC, ACV-PC/PG, and CAM-PC were performed with a Malvern Zetasizer Nano Z (Malvern, Worcestershire, UK). The liposome dispersions were diluted 1:40 in MilliQ water prior to the measurements to achieve proper count rates, and the results reported were obtained from at least three measurements.[17]

Entrapment Efficiency Determination

To separate free drugs from liposomally entrapped drugs, extruded liposomes were centrifuged in a Beckman model L8–70M ultracentrifuge with an SW 60 Ti rotor (Beckman Instruments, Palo Alto, California). The samples were centrifuged at 216,000*g* for 60 min at 10°C. Pellets were resuspended in distilled water and further diluted in methanol to dissolve the lipids before the concentration of CAM or ACV was quantified by HPLC as described below. The supernatants were measured by PCS to verify that they contained no vesicles and then diluted in methanol before quantification by HPLC. The experiments were performed in triplicate, and the entrapment efficiency was expressed as the drug/lipid ratio.

Quantifications of ACV, CAM, CF, and Calcein

The concentrations of ACV and CAM were determined by HPLC. A reversed-phase column (Waters XTerra® C18; 5 μm; 3.9 × 150 mm^2; Waters, Milford, Massachusetts) installed in a Waters e2795 separations module equipped with a UV 2489 detector was used. Wavelengths of 258 and 280 nm for ACV and CAM, respectively, were used.[17,25] Mobile phase for ACV detection was MeOH/MilliQ water 50:50, pH 2.5 (HCl), and that for CAM was MeOH/MilliQ water 45:55, pH 2.5 (glacial acetic acid).[17,25] The run time was 7 min, and the sample injection volume was 10 μL. The column temperature during the quantification of ACV was 25°C and it was 30°C for CAM.