INDONESIA INTERNATIONAL INSTITUTE FOR LIFE SCIENCES

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PROTOCOL

In Vitro Percutaneous Absorption Study of Penetration Enhancers in Cosmetic Formulation Towards Active Ingredient Against the Skin Models

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The authors wrote this protocol handbook in hopes of guiding users conducting studies involving *in vitro* percutaneous absorption methods. The protocols written in this handbook were initially developed to study the percutaneous absorption of penetration enhancers, particularly in cosmetic formulations, towards active ingredients using a variety of *in vitro* skin models. The authors believe that this handbook would provide insight for similar areas of study.

Date : 11 May 2022

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1. INTRODUCTION

Human skin is the largest organ that covers 16% of the body weight. Normally, skin comprises a multilayer membrane that is known as hypodermis, dermis, and epidermis (Kahraman, et al., 2019). Stratum corneum (SC) is known as the outermost layer of epidermis. It is made up from the binding interaction between corneocytes and lipid interface which includes free fatty acids, cholesterol, and ceramides (Elias & Wakefield, 2010). Stratum corneum also works as the permeability barrier and antimicrobial defenses. In general, the mechanism of barrier function as the permeability is by the regulation of IL-1α, Ca++, pH, liposensors, serine proteases signaling through the PAR2, TRPV1 and 4 as the receptors. Meanwhile, the antimicrobial activity of the skin barrier is regulated by the signaling of 1,25 (OH)2D3, an IL-1α. The signaling pathway of SC as the permeability barrier and antimicrobial defense are occured in lamellar bilayer (Elias, 2008). Moreover, skin is known as the important route for topical and systemic administration (Praça, et al., 2018).

Skin naturally is able to regulate the passage of a certain compound to pass through the skin layer, scientifically known as percutaneous absorption. In general, percutaneous absorption is classified into three steps, starting from penetration, permeation, and resorption (Bartosova & Bajga, 2012). Penetration is known as the entry of a particular substance to the SC. Following that, the compound is penetrated from the SC to a deeper layer which has a different function and structure. This process is called permeation. This step is carried out by a diffusion process. The skin permeation is known to be slower than penetration due to the binding of various types of ions (e.g. metal ions, ammonium ions), sulfonium salts, acrylates, etc. SC is mainly used as the rate limiting barrier due to the high resistance to diffusion rate. Meanwhile, resorption is known as the process of uptake of a particular substance into a vascular system.

The chemical transport into the skin involves three mechanisms (Bartosova & Bajga, 2012). Transcellular absorption is the process when the particular compound is transferred throughout the keratin-packed corneocytes by a cell membrane partitioning. The mechanism is followed by the intercellular absorption pathway. It occurs when the chemical is moving around the corneocytes which happens in lipid-rich extracellular regions. The end of the second pathway is indicated by the chemicals bypassing the corneocytes and entering the shunts that are provided by the hair follicles, sebaceous glands, and sweat glands. In the percutaneous absorption, a compound that is contacted with skin is generally crossed the diffusional barrier, followed by the uptake by the capillary network that is intended for systemic circulation. However, the compound might undergo evaporation from the surface of the skin. The binding and penetration to SC also possibly occurred as well as the compound is able to be metabolized.

Due to the external and internal environment, skin faces some challenges that might decrease its properties. Cosmetics appear as the solution to overcome many skin problems. It is defined as a preparation that is used to clean, beautify, promote attractiveness, alter the body appearance, and also maintain the skin and hair condition (Misui, 1997). Cosmetics are generally classified into skin care, hair care, oral care, and body cosmetics with various pharmacological activities such as anti-aging,

anti-wrinkle, whitening agent, cleansing agent, etc. It has been proven that the presence of an active ingredient in cosmetics is expected to exert protective activity, resulting in an improvement of the skin (Kraeling, et al. 2015). However, many of the active ingredients have limited penetration due to many factors, resulting in reduced activity. Therefore, to ensure adequate activity, percutaneous absorption study of the penetration enhancers are important to determine the ability of the penetrant to stimulate the API to penetrate into a deep layer.

Percutaneous absorption study is an evaluation of the ability of a certain ingredient to pass through the upper skin membrane. Penetration study is commonly carried out either by *in vitro* or *in vivo* test. *In vitro* penetration test is carried out by applying the cosmetic formulation into multiple types of either human or animal skin models. It is designated to measure the ability of chemicals to cross the skin membrane into a fluid reservoir. It is appropriately used to predict human dermal penetration study. *In vitro* method is preferred due to a lower cost, time needed, reproducibility, and less restricted parameters.

AIM OF STUDY : to study the effects of penetration enhancers towards the API in a cosmetic formulation through *in vitro* penetration testing by using MatTek Permeation Fixture (EPI-200-FIX)

2. MATERIALS AND METHODS

a. *In vitro* model/Subject

- Device : MatTek Permeation Fixture (EPI-200-FIX)
- Skin Model : EpiDerm™ Skin Model (EPI-200-X)
- Buffer : Phosphate Buffer Saline (PBS) pH 7.4
- Donor Solution : Liquid or semi-solid dosage form for topical use

b. Test Compound (Groups of study or experimental design)

Test compound	Storage
Negative Control (No Penetrant)	Room Temperature, Protect from light
Base Value (Control Positive)	
Sample	

c. Methods

c.1 Method origin

Prior to the penetration testing, the calibration curve of the API should be constructed in order to find the Limit of Detection (LOD) and Limit of Quantification (LOQ) to define the lowest concentration of the API that can be detected and quantified (Kumar, *et al.,* 2018). The *in vitro* percutaneous absorption study is conducted with Mattek Permeation Device (MPD) to determine the effect of penetration enhancers towards the API (). The method was mostly developed by Mattek Company with some adjustment for semi-solid dosage form. PBS solution pH 7.4 was chosen due to its abundant availability and numerous studies of penetration testing using PBS.

c.2 Method Principle

The principle of the method is similar to Franz-diffusion cells, where the donor solution is inserted to the device equipped with a membrane. The API is expected to pass through the membrane and fall into a receiver solution. To prove the presence of the API, the receiver solution is analyzed through the UV-Vis spectrophotometer at the selected wavelength to obtain the absorbance. The data then will be processed with the selected formula to find the cumulative drug release and the average flux of the API to penetrate. The experiment was conducted in three biological and technical replicates.

c.3 Description of the method

c.3.1. Calibration Curve of API

1. Make a stock solution with Distilled Water (DW) Type III as a buffer
2. Dilute the stock solution by doing a serial dilution
3. Test the sample with UV-Vis Spectrophotometer. Prior to analysis, find the excitation wavelength of the API.
4. Make a standard calibration curve and find the equation and r value should > 0.995
5. Find the standard deviation, LOD, and LOQ

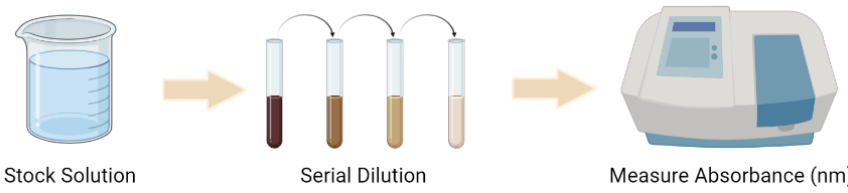

Figure 1. General procedure for calibration curve of API

c.3.2. *In vitro* Percutaneous Absorption Test

c.3.2.1. Epiderm Pre-Treatment

1. Prior to the following steps, a biosafety cabinet (BSC) should undergo UV sterilization for 5-10 minutes and all surfaces along with the equipments are wiped using 70% ethanol
2. The MatTek assay medium is pre-warmed to 37°C and 900 µl of the medium is pipetted into a clean 6-well plate using sterile technique

3. Open the Epiderm packaging under sterile conditions and transfer the Epiderm inserts into 6-well plates containing the MatTek assay medium using sterile forceps
4. Incubate the Epiderm in a humidified 37°C, 5% CO_2 incubator overnight prior to treatment

c.3.2.2. MatTek Permeation Fixture (EPI-200-FIX) Set Up

1. Remove 3 screws from the permeation fixture and take out the top piece
2. Using sterile forceps, insert the prepared Epiderm onto the bottom piece
3. Replace the top piece to keep the Epiderm snugly in between the fixture pieces
4. Return the screws and tighten properly
5. After used, EPI-200-FIX is able to be reused by soaking the device into the ethanol 70% for 30 minutes. *(Notes: Don't autoclave the device since it cause a deformation of EPI-200-FIX)*

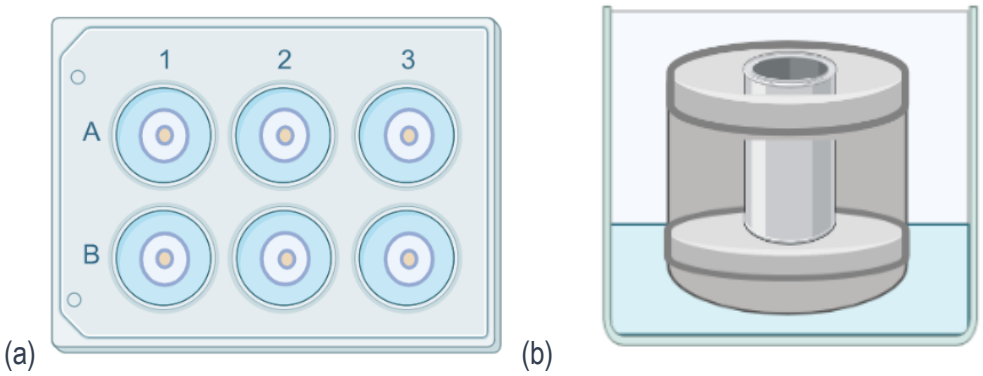

Figure 2. Illustration for Mattek Permeation Device (MPD) setting (a) top view (b) side view

c.3.3.3. Penetration Testing

1. Put the Mattek Permeation Fixture (EPI-200-FIX) in the six well plates and put the skin models appropriately. Ensure that the whole procedure is conducted in the BSC (*Notes: Epiderm skin model is needed to be dipped in the PBS for 30 mins - 1 hour)*
2. Add the PBS solution pH 7.4 to each well
3. Insert the donor solution to the EPI-200-FIX that is already set with the membrane. Dilute the donor solution 20x with Deionized (DI) water before the analysis in order to obtain a good absorbance *(Notes: for semi-solid dosage form or highly viscous liquid, the donor solution is added directly to the membrane before the top piece is set and tighten properly with screw to prevent the donor solution stuck on the wall of the top piece of EPI-200-FIX)*
4. Take the whole receiver solution and replace with a new PBS solution at the following time points: 0.5, 1, 2, 4, 6, and 8 hours *(Notes: for semi-solid dosage form or highly viscous liquid, dilute the receiver solution 20x with PBS pH 7.4 before the measurement).* Save the solution for further analysis
5. Make a standard curve with PBS pH 7.4 as a buffer for the further data analysis

6. Measure the solution with UV-Vis Spectrophotometer in order to obtain the absorbance of the withdrawn receiver solution

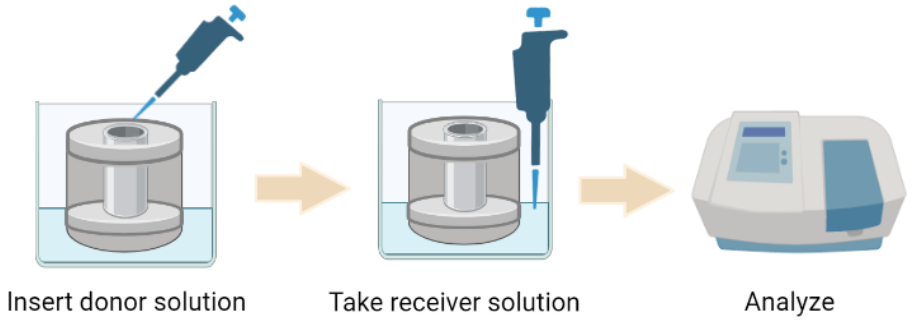

Figure 3. General procedures of penetration testing

c.4 Materials and Equipment used

1. Materials
    a. EpiDerm™ Skin Model (EPI-200-X)
    b. Phosphate Buffer Saline (PBS) pH 7.4
    c. Distilled Water (DW) Type III
    d. Deionized (DI) water
    e. 6 well-plates
    f. Sample or donor solution

2. Equipments
    a. UV-Vis Spectrophotometer
    b. Biosafety cabinet (BSC)
    c. MatTek Permeation Fixture (EPI-200-FIX)
    d. Humidified 37°C, 5% CO_2 incubator

3. DATA COLLECTION AND STATISTICAL ANALYSIS

Calibration Curve of API

1. Calculate the LOD and LOQ (ppm):

Limit of Detection (LOD) = 3.3 x (SD of intercept/slope)

Limit of Quantification (LOQ) = 10 x (SD of intercept/slope)

Penetration Testing

1. Calculate the amount of Drug Release

$$Amount\ of\ Drug\ Release\ (ug) = \frac{Concentration\ of\ sample\ (ug/ml)\ x\ Total\ receiver\ solution\ (ml)\ x\ Dilution\ Factor}{Total\ amount\ of\ API\ in\ the\ donor\ solution\ (ug)}$$

2. Find the cumulative drug release

$$Cumulative\ Drug\ Release\ (\%) = \frac{Total\ sample\ withdrawn\ (ml)}{Total\ bath\ volume\ (ml)}\ x\ p(t-1) + p(t)$$

3. Find the average flux

$$Average\ Flux\ (ug/hr) = \frac{Amount\ of\ drug\ release\ (ug)}{Time\ (hr)}$$

4. The cumulative drug release and average flux are calculated at each time points and the data should be depicted in the form of graph to visualize the result

Statistical Analysis

Penetration Testing is conducted in triplicates. Graph plotting and statistical analysis were performed using GraphPad Prism 8.0.1. The statistical analysis is carried out with one-way ANOVA analysis, with Post-hoc tests conducted to identify significant differences between groups.

4. RESULT INTERPRETATION

Penetration Testing

a. Cumulative Drug Release

Cumulative drug release is calculated to determine the percentage of API that is passed through the membrane. The higher percentage of drug release of the samples compared to the control negative indicates that the penetration enhancer works to improve the penetration profile of the API.

b. Average Flux

Average flux is calculated to analyze the flux of the API penetrated over time. Commonly, the formula achieves the highest flux at the first few hours due to a different concentration between donor and receptor compartment. As the time passes by, the flux will decrease gradually and reach the steady state since the concentration of both donor and receptor reaches equilibrium.

5. REFERENCES

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6. SUPPLEMENTARY DATA, TABLES AND FIGURES

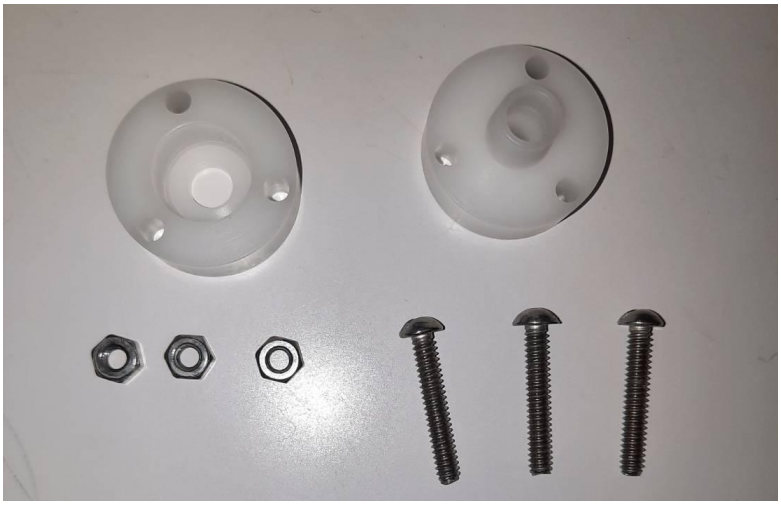

Figure 1. MatTek Permeation Fixture (EPI-200-FIX) parts. Parts include a top piece with protruding annulus (tube) and bottom piece with a chamber, along with 3 screws and nuts.