ABSTRACT

Cosmetics containing rhododendrol (RD) were voluntarily recalled after incidents of leukoderma related to their use. Users reported using up to five different RD products by layered application. It was hypothesized that layered application increased the skin permeation of RD, resulting in leukoderma. The role of tyrosinase inhibition and melanocyte cytotoxicity of RD was implicated, however, from a pharmaceutical point of view, these provide limited insights on the influence of formulations, and in-use conditions on skin permeation of RD.

In the 1st Chapter, we investigated the effects of layered application, formulations, and their components on the skin permeation of cosmetics containing RD. Experiments were designed to simulate actual in-use conditions, such as varying application volumes, physical mixing of formulations, sequence of cosmetics application and time interval between applications, to establish their effect on permeation. Milk and lotion RD-containing cosmetics (2%), 1% aqueous RD, and preparations of formulation components were applied as the first or second layers as finite doses of 10 or 20 µL/cm^2. Permeation experiments were performed through excised porcine ear skin using Franz diffusion cells. Cosmetics applied by layered application exhibited lower skin permeation of RD compared with a single application despite having the same application dose. High initial volume (20 µL at 0 or 5 s) did not exhibit any significant reduction in the permeation of RD. Formulations and their components reduced RD permeation, probably due to changes in thermodynamic activity of the active component. Layered application, formulation components, application volume, time interval and sequence of application had significant influences on the skin permeation of the active component.

Rapid evaporation of solvents occurs from topically applied formulations in finite dose systems which alters the vehicle composition. The finite dose experiment represents clinical use wherein depletion of dose and evaporation of excipients may occur. In the 2nd Chapter, we attempted a mathematical approach for predicting skin permeation and concentration of RD, from complex vehicle-based formulations applied as finite dose. *In vitro* skin permeation and concentration studies of RD were conducted from formulations containing water and polyols with concentrations ranging from 10 – 100% under infinite and finite dose conditions. Observed data for skin permeation and the viable epidermis and dermis (VED) concentration of RD were estimated by the differential equations under Fick's second law of diffusion together with water evaporation kinetics and changes in the partition coefficient from vehicles to the stratum corneum. As a result, a goodness-of-fit was observed allowing accurate estimation of skin permeation and VED concentration of RD.

Finally, we investigated the effects of layered application and other finite dose conditions using an artificial membrane, Strat-M®. The use of artificial membranes designed to mimic animal skin offer a competent alternative to estimate skin permeation. However, its usefulness in the assessment of permeation from complex formulations under in-use conditions has not been clarified. Assessment of dermal absorption is ascribed to be performed using porcine skin, hence, it is imperative to establish the equivalency of Strat-M®. Permeation of drugs from formulation of high polyol content and residual formulation is increased with an increase in the permeability of the artificial membrane. Barrier integrity of Strat-M® is disrupted by high concentration of polyol as evidenced by reduction in electrical impedance. The use of Strat-M® in the assessment of dermal permeation may be limited to finite dose conditions and not in concurrent application of formulations and infinite dose conditions.

GENERAL INTRODUCTION

Functional cosmetics typically contain an active component (quasi-drug) that serves as the basis for their marketing claims. Exposure to cosmetic active components could induce forms of localized skin toxicities. In recent times, cosmetics containing rhododendrol (RD) were voluntarily recalled after incidents of leukoderma related to their use.[1] Users reported using up to five different RD-containing products concurrently suggesting a link between the incident and the applied dose and cosmetics use habits.[2] RD was shown to exhibit melanocyte cytotoxicity at high concentrations. [3-6] However, from a pharmaceutical point of view, it provides very limited insights into the influence of formulations and the manner of cosmetics application on the skin permeation of actives. Evaluation methods based upon appropriate skin models and in-use conditions could confirm the dose-dependent toxicity of compounds at the site of action. [7-12] The efficacy and safety of cosmetics and locally acting drugs applied on skin are determined by their distribution into its intended site of action, the viable epidermis and dermis (VED). [13-18]

Numerous reports have described techniques to assess the permeation of cosmetic active compounds through the skin. [18-22] Evaluation of dermal permeation is typically conducted under finite and infinite experiments. Finite dose experiment is supposed to best represent its clinical use (i.e., in-use conditions) wherein depletion of dose and evaporation of excipients may occur. On the other hand, an infinite dose experiment is characterized by a non-depleting dose and allows estimation of permeation parameters. Under in-use conditions, rapid evaporation of solvents occur which significantly alter the effective diffusion area of the applied formulation and the composition of the resulting residual formulation after formulations are applied on the skin. [23-25] The impact of vehicle dynamics on the skin permeation can be realistically

clarified by simulating the residual formulation based on evaporation kinetics from applied formulations. To estimate dermal absorption, experimental conditions should be as close as possible to real exposure conditions reflecting in-use conditions such as the use of finite dose, and periods of exposure. [26-27] Similarly, the appropriate conduct of *in vitro* dermal absorption studies must encompass dose, and vehicle/formulation conditions should represent the in-use conditions. Experimental conditions for *in vitro* dermal absorption studies of cosmetics for dose or amount applied during use (i.e., layered application), formulation (e.g., finished cosmetics products, complex vehicles), and barrier integrity must be met. [20-23]

Methods to assess dermal permeation include mathematical models aimed at predicting skin or VED concentration of chemicals. It entails the understanding of the factors (e.g., diffusion and partition coefficient, solubility parameters) that influence skin permeation. [18,28-32] The inclusion of vehicle dynamics is an approach viewed to enhance the accuracy of mathematical models in predicting skin or VED concentration from cosmetic formulations. Another method employed to estimate permeation of drugs and safety assessments is the use of skin membranes and artificial membranes (e.g., Strat-M®, silicone membrane). [33-40] However, the usefulness of artificial membrane, Strat-M®, has not yet been verified in the context of cosmetics in-use conditions. Assessment of dermal absorption of cosmetic actives is ascribed to be performed using porcine skin as it resembles closely human skin properties such as permeability to chemicals, thickness and lipid composition. [41-43] Hence, being the membrane of choice, it is imperative to understand the similarities and establish equivalency and relationship between Strat-M® and porcine skin in terms of membrane characteristics confirm its applicability in evaluating permeation of cosmetic actives.

CHAPTER 1

Effect of layered application on the skin permeation of a cosmetic active component, rhododendrol

1.1 Introduction

Cosmetics containing RD were voluntarily recalled from the market after incidents of leukoderma related to their use. Users who experienced RD-induced leukoderma reported using up to five different RD-containing products concurrently suggesting a link between the incident and the applied dose of cosmetics. [2] Habits related to cosmetics use along with the amount of cosmetics applied may have predisposed users to product-use related toxicities. Sasaki *et al*. reported that RD exhibits cytotoxicity against cultured human melanocytes at high concentrations. [3] In fact, skin permeation and skin concentration of topically applied drugs and cosmetics often determine their efficacy or toxicity. [16] It was hypothesized that layered application of RD, that is, increasing the number of applied products on the skin, increased the skin permeation of RD, resulting in leukoderma. [1] Several studies attempted to clarify the cause of leukoderma and suggested the role of tyrosinase inhibition and melanocyte cytotoxicity of RD. [44-46] From a pharmaceutical point of view, these results provide very limited insights into the influence of formulations and their components, and the manner of cosmetics application (i.e., single or layered application, sequence of product application, application time interval, etc.) on the skin permeation of cosmetic active components.

Layered application is described as the application of a second or succeeding dose (layer) of cosmetics on the same region after an initial application. Quasi-drug formulated as medicated cosmetics are pharmacologically or cosmetologically active, and they are commonly sold in sets to elicit their purported effects synergistically.

These cosmetics are in fact recommended to be applied sequentially and in layers. Moreover, cosmetics have additional esthetic requirements of the active components and vehicles where excipients are added for reasons unrelated to dermal permeation yet may have effects on the penetration of the active components. [47]

A previous work revealed that RD permeation after layered application resulted in a dramatic decrease in its permeation. [1] Cumulative amounts of RD permeated in infinite doses (1.0 mL/1.77 cm^2) of aqueous RD was much higher than those of finite doses (10 and 20 µL/cm^2) due to depletion of RD in finite dose models. Interestingly, layered application (20 µL/cm^2 × 2) of RD in a lotion formulation resulted in lower permeation than a single application (40 µL/cm^2) despite having the same total volume applied. The mechanistic explanation on how layered application of RD cosmetics could cause a decreased permeation profile and leukoderma remains unresolved. Although numerous studies on cosmetics safety and testing procedures have been performed, [48-49] the safety of practicing layered application as in most cosmetics and topical drugs have never been investigated before. Furthermore, no studies have evaluated the actual manner (layered application) in which consumers use these medicated cosmetics. Also, there are no studies clearly depicting a mechanism on how actives would permeate following layered application.

Recently, actual consumption of cosmetic products reflecting Japanese cosmetics habits has been reported, prompting the need to conduct risk assessments of cosmetics products and their reported consumption dose. Also, the reported habit of using up to 5 different RD products simultaneously indicated that the amount of cosmetics consumed may be a predisposing factor for its toxicity. [2] In addition, changes in skin permeation of cosmetic active components as influenced by the manner of application should be considered in the development of cosmetic formulations.

In order to accurately assess the safety of chemical substances, it is important to simulate exposure as realistically as possible. Hence, in the present study, experiments were designed to simulate actual in-use conditions and multiple "practices" such as varying sequences of cosmetics application, layered application, varying application volumes, and time intervals between applications to establish its effect on the skin permeation of cosmetic active components. In addition, the effects of formulations and their components on the skin permeation of cosmetic active component (RD) were also investigated.

1.2 MATERIALS AND METHODS

1.2.1 Chemicals

Rhododendrol (CAS no. 501-96-2; ≥99%) (Fig. 1) was provided by Kanebo Cosmetics Inc. (Tokyo, Japan). Methyl paraben, glycerin, dipropylene glycol (DPG), and sorbitol were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). 1,3 - Butylene glycol (BG) was purchased from Tokyo Chemical Industry, Co. Ltd. (Tokyo, Japan) and propylene glycol (PG) was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Previously marketed and recalled RD products (lotion and milk, 2%) were provided by Kanebo Cosmetics Inc. (Tokyo, Japan).

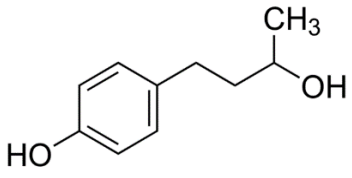

Figure 1. Structural formula of rhododendrol

Table 1. Physicochemical properties of RD

Parameters	Values
Molecular weight	166.22
Clog*P*	1.9
p*K*a	6.2
logK_{ow}	1.4

1.2.2 Preparation of rhododendrol formulations

Table 2 shows the primary components of RD cosmetic formulations. Aqueous RD solution (1%) was prepared by dissolving a sufficient amount of RD in purified water to reach the desired concentration. Aqueous RD solution (1%) was prepared instead of 2% owing to its poor solubility. Lotion containing no RD was prepared using primary components, glycerin, DPG, BG, water, and sorbitol at defined proportions. A physical mixture of milk and lotion cosmetics was prepared by mixing equal amounts of milk and lotion using vortex mixer for 5 min prior to application.

1.2.3 RD solubility studies

Solubility of RD in each primary component (water, 5 and 10% BG solution, BG, DPG, glycerin, sorbitol, PG) was determined by adding excess amount of RD with constant stirring at 30°C for 24 – 48 h. The preparation was filtered using a polytetrafluoroethylene membrane syringe filter (Advantec®, 0.2 μm, Tokyo, Japan) and the obtained filtrate was injected into an HPLC system to determine the concentration of RD.

Table 2. Primary components of RD cosmetic formulations

Components	Lotion	Milk
Glycerin	+	+
Dipropylene glycol (DPG)	+	+
1,3-Butylene glycol (BG)	+	+
Water	+	+
Sorbitol	+	+
Propylene glycol (PG)	-	+

** Concentration of formulation components was not indicated with the intent of Kanebo Cosmetics Inc. and Kao Corporation.*

1.2.4 In vitro skin permeation experiment

Frozen edible porcine ears (Central Institute for Feed and Livestock, JA Zen-Noh, Ibaraki, Japan) were thawed with warm water (32°C) and rinsed with purified water. Hairs were shaved off and excess subcutaneous fats were trimmed off from the excised intact skin. Excised skin of similar thickness was derived from the same region (central dorsal) of the porcine ear. Prior to the excision of skin, visual inspection was performed to ensure intact and damage free skin was utilized. The prepared porcine ear skin was directly set on a vertical-type Franz diffusion cells with an effective diffusion area of 1.77 cm^2 and the skin surface temperature was thermostatically maintained at 32°C. Hydration was done by application of 1 mL purified water to the epidermis side and 6 mL purified water was applied to dermis side (receiver compartment) to reach an equilibration state for 1 h. Purified water on the epidermis side was removed and excess water was blotted with cotton swab. Then, RD formulation was applied with a micropipette (see Table 3). Sampling was performed by withdrawing 500 µL of receiver solution every hour for 8 h. Samples were analyzed by HPLC.

1.2.5 Experimental design for layered application

Factors such as formulations and other preparations investigated in the permeation experiments are described in this section. The sequence of application (which was applied as the first or second layer), the interval between application of the layers and application volume are also presented. The application time (5s, 5 or 10 min) for the second layer applied is based on the time after the application of the first layer at t_0.

Study codes for applied formulations. The factors investigated are represented using specific study codes. As a case in point, L-L_{10B}, refers to 10 µL of

lotion applied as the first layer onto the prepared porcine ear skin followed by a second layer of 10 µL of lotion after 5 min. The first code (i.e., L, So, etc.) denotes the formulation of the first layer followed by a "-" symbol denoting layered application, then the code for the second layer applied. Applications utilizing mixtures of milk and lotion include a "+" symbol. Application volumes of 10, 20, and 40 µL are indicated by 10, 20 and 40 in subscript format, respectively. The application time interval between layers is indicated by letters - A for 5 s, B for 5 min, and C for 10 min, also in subscript format. Non-layered or single applications include the letter "s". Complete study codes on the factors investigated are listed in Table 3.

Application volume. RD preparations (2% RD lotion and milk formulations, 1% aq. RD) and formulation components were applied as finite doses. For layered application, 10 and 20 µL/cm^2 volumes were used as application doses for the first and second layers of cosmetics in the experiments. Single application experiments were carried out at either 20 or 40 µL/cm^2.

Sequence of application and physical mixture. In actual use, the application of cosmetics follows a defined sequence as to which formulation is applied first. Lotion is often recommended to be applied as a base cosmetic or first layer, whereas milk is used to a lesser extent. In the present experiment, milk and lotion formulations were applied either as the first or second layers. Table 3 summarizes the experimental design for the layered application of cosmetics.

Application time interval. Application time interval for layered application was observed at 5 s, 5 min, and 10 min. The intended interval time was allowed to elapse prior to the application of the second layer of cosmetics. Actual interval time of application among consumers has not been reported, hence, 5s, 5 min and 10 min application intervals were arbitrarily selected to reveal its effect on RD permeation.

Applied formulations were spread using a spatula over the effective permeation area to ensure uniform distribution.

Formulation components. Effect of formulation and individual components on RD permeation was also investigated using identical layered application experiments to those described above. DPG, BG, PG, sorbitol, glycerin and water were the formulation components investigated in the present study. Lotion formulation containing no RD was also prepared. Formulation components were applied as the first or second layer together with lotion or 1% aq. RD.

Table 3. Experimental design for layered application of RD lotion and milk

a		L-L_{10A}	L-L_{10B}	L-L_{10C}	L-L_{20A}	L-L_{20B}	Ls	M-M_{10A}	M-M_{10B}	M-M_{10C}	M-M_{20A}	M-M_{20B}	Ms
1st layer applied	lotion	+	+	+	+	+	+						
	milk							+	+	+	+	+	+
2nd layer applied	lotion	+	+	+	+	+							
	milk							+	+	+	+	+	
Application volume	10 µL	+	+	+				+	+	+			
	20 µL				+	+	+				+	+	+
Application interval	5 s	+			+		N/A	+			+		N/A
	5 min		+			+			+			+	
	10 min			+						+			

b		L+M - L+M_{10A}	L+M - L+M_{20A}	L+Ms_{10}	L+Ms_{20}	L+Ms_{40}	L-M_{10A}	L-M_{10B}	M-L_{10A}	M-L_{10B}
1st layer applied	lotion						+	+		
	milk								+	+
	L+M*	+	+	+	+	+				
2nd layer applied	lotion			N/A	N/A	N/A			+	+
	milk						+	+		
	L+M*	+	+							
Application volume	10 µL	+		+			+	+	+	+
	20 µL		+		+					
	40 µL					+				
Application interval	5 s	+	+	N/A	N/A	N/A	+		+	
	5 min							+		+
	10 min									

**L+M: physical mixture of RD lotion and milk*

c		R-R_{10B}	(-)RD-L_{10B}	So-L_{10B}	W-L_{10B}	4B-L_{10B}	DP-L_{10B}	G-L_{10B}	W-R_{10B}	R-$4B_{10B}$	4B-R_{10A}	BG-R_{10B}	R-BG_{10B}
1st layer applied	1% aq. RD	+								+			+
	Lotion w/out RD		+										
	Glycerin							+					
	DPG						+						
	4% BG					+					+		
	Sorbitol			+									
	BG											+	
	Water				+				+				
2nd layer applied	1% aq. RD	+							+		+	+	
	Lotion		+	+	+	+	+	+					
	4% BG									+			
	BG												+
Application volume	10 µL	+	+	+	+	+	+	+	+	+	+	+	+
	20 µL												
Application interval	5 s										+		
	5 min	+	+	+	+	+	+	+	+	+		+	+
	10 min												

Legends:
L, M, (-)RD, R, and W: lotion, milk, 1% RD aqueous solution, and water
4B, BG, G, So, and DP: 4% 1,3-butylene glycol (BG), 100% BG, glycerin, sorbitol, and dipropylene glycol (DPG)
Subscript format 10, 20, 40: application volume (∝L)
Subscript format A, B, and C: 5s, 5 min and 10 min for application interval time between layers
-, +, and s: layered application, physical mixture and single application
N/A: not applicable

1.2.6 HPLC Analysis

Samples (100 µL) were added with an equal volume of acetonitrile containing the internal standard (methyl paraben) and centrifuged at 4°C for 5 min. Each sample was analyzed using an HPLC system (Shimadzu Co., Kyoto, Japan) consisting of column (Inertsil® ODS-3 4.6 mm X 150 mm, GL Sciences Inc., Tokyo, Japan), system controller (SCL-10A), pump (LC-20AD), degasser (DGU-20A_3), auto – injector (SIL-20A), column oven (CTO-20A), UV detector (SPD-20A), and analysis software (LC Solution). The column was maintained at 40°C with a flow rate of mobile phase (acetonitrile: water = 25:75) at 1.0 mL/min. Detection of RD was made at 280 nm.

1.2.7 Measurement of transepidermal water loss (TEWL) at application site

Measurement of water loss at each time-point was performed to estimate cumulative amount of water which evaporated from the applied formulation. Evaporation of water from applied formulation (10 µL/cm^2) on porcine ear skin was monitored using Vapo Scan (AS-VT100RS, Asahi Techno Lab., Yokohama, Japan) over a 20 min observation period. The TEWL measurement was performed at ambient temperature (20- 25°C) and RH of 30 % ± 2.

1.2.8 Statistical Analysis

Experimental data on the cumulative amount of RD permeated were tested for statistical significance ($p < 0.05$) using one-way ANOVA and Tukey's HSD post hoc analysis. Water loss data were tested for statistical significance ($p < 0.05$) using Student's *t*-Test.

1.3 Results

1.3.1 Effect of layered application, volume, and time interval between applications on the skin permeation of RD

Figures 2A and B show the effect of interval time in layered application of either lotion or milk formulation, respectively, on the skin permeation of RD. When 40 µL of lotion in total was applied in portions (20 µL × 2, layered application) at time intervals of 5 s and 5 min, almost the same permeation profile was observed which was approximately two-fold higher compared with a single application of 20 µL lotion (Fig. 2a). In contrast, a lower RD permeation profile was observed with the layered applications of 10 µL lotion (10 µL × 2) with increasing interval time of 5 s, and 5 and 10 min, accordingly (Fig. 2A). With prolongation of time interval between application, a greater reduction in RD permeation was observed. A similar tendency was observed in RD permeations from milk formulations (Fig. 2B). However, the decrease in the RD permeation from milk formulations (Fig. 2B) was less than those from lotion applications (Fig. 2A).

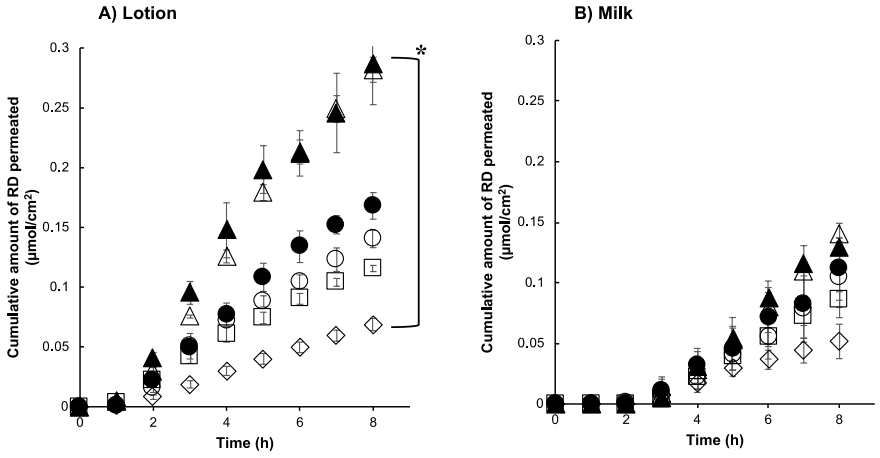

Figure 2. Effect of layered application, interval time and initial application volume of lotion (a) and milk (b) formulations on the skin permeation of RD. a) L-L_{10A} (○), L-L_{10B} (□), L-L_{10C} (◊), L-L_{20A} (▲), L-L_{20B} (△), Ls (●); b) M-M_{10A} (○), M-M_{10B} (□), M-M_{10C} (◊), M-M_{20A} (▲), M-M_{20B} (△), Ms (●). Each value represents the mean ± S.E. (n = 3 – 5). *$p < 0.05$. Study code L-L_{10A} refers to layered application of 10 µL lotion with 5 s interval time of application prior to the second application of lotion (10 µL). A, B and C represents interval time of application 5 s, 5 min and 10 min, respectively. Complete details in Table 3 and section 2.4.

Skin permeation of RD was then evaluated from physical mixture of lotion and milk. The physical mixture corresponds to layered application with an interval time of 0 s. Figure 3 shows the results. For the physical mixture of lotion and milk, larger volumes (> 20 µL) applied at the beginning (within 0-5 s) did not result in a reduction in RD permeation compared with a single application of the physical mixture.

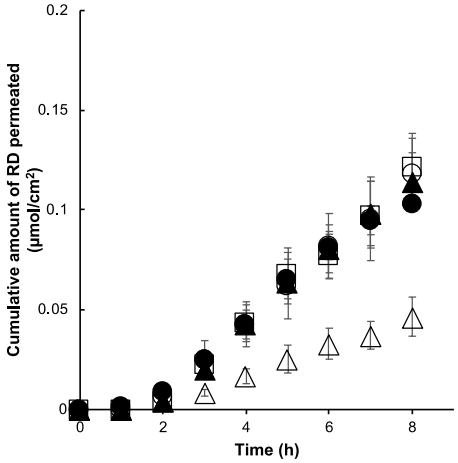

Figure 3. Effect of initial application volume of the physical mixture of lotion and milk on the skin permeation of RD. L+M - L+M_{10A} (○), L+M – L+M_{20A} (▲), L+Ms_{10} (△), L+Ms_{20} (●), L+Ms_{40} (□). Each value represents the mean ± S.E. (n = 3-5). Study code L+M - L+M_{10A}, refers to layered application of physical mixture of lotion and milk (L+M) and L+M with 5 s interval time of application. L+Ms refers to single application of physical mixture of lotion and milk. Complete details in Table 3 and section 2.4.

1.3.2 Effect of sequence of cosmetics application (lotion to milk and vice versa) on the skin permeation of RD

Figure 4 shows the skin permeation of RD after application of lotion or milk as the first layer prior to the addition of the second layer of milk or lotion, respectively. A physical mixture containing equal amounts of lotion and milk was applied as the first layer and served as the basis for comparison. The skin permeation of RD after the application of lotion as the first layer and milk as the second layer and vice versa, with a 5 s interval time exhibited a lower skin permeation compared with the physical mixture. A similar pattern was noticed wherein longer time interval between

applications resulted in lower RD permeation in experiments using lotion as the first layer and milk as second layer, and vice versa. Milk applied as first layer exhibited lower permeation as compared with lotion being applied as the first layer.

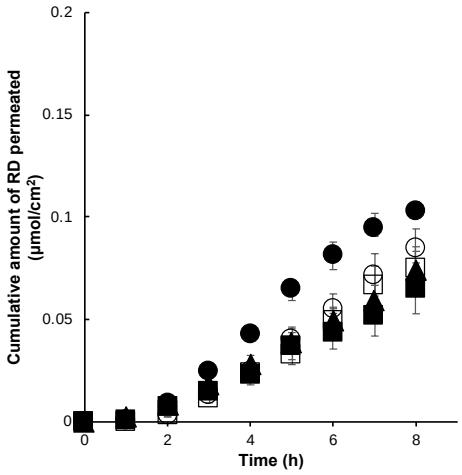

Figure 4. Effect of sequence of application (lotion to milk and milk to lotion) on the skin permeation of RD. L-M_{10A} (○), L-M_{10B} (□), M-L_{10A} (▲), M-L_{10B} (■), L+Ms_{20} (●). Each value represents the mean ± S.E. (n = 3-5). Study code L-M_{10A} refers to layered application of lotion as first layer and milk as second layer with 5 s interval time of application. Complete details in Table 3 and section 2.4.

1.3.3 Effect of cosmetic formulations and formulation components on the skin permeation of RD

Figure 5a shows the effect of formulation on the skin permeation of RD when applied in layers. Layered application of aqueous solution containing 1% RD exhibited a 3.4-fold higher permeation compared with its formulated counterparts containing 2% RD, even at a half concentration of RD. It was noticeable that the skin permeation of RD from milk and lotion formulations was significantly lower. This indicated that the type of formulation markedly affected the skin permeation of RD.

Both the lotion and milk formulations used in the present study contained glycerin, DPG, BG, water and sorbitol as the primary formulation components (Table 2). Thus, the effect on the skin permeation of RD by pretreatment with these primary components when applied as the first layer was evaluated. Figure 5B shows the effect

of pretreatment with primary components applied as the first layer on RD permeation. Formulation of lotion without RD (containing primary components only) was applied as the first layer and followed by application of lotion with 5 min interval resulted to the greatest reduction in the skin permeation of RD. Higher RD permeations were observed when sorbitol and water were applied as the first layers. On the other hand, decreased RD permeation was observed when BG, DPG and glycerin were applied as the first layers compared with sorbitol and water.

Figure 5C shows the skin permeation of RD for the two cases of treatment. An aqueous solution of RD was applied as the first layer followed by application of the same aqueous RD solution, 4% BG or BG for the first case. Aqueous RD solution, water or BG solution was applied as the first layer and followed by application of aqueous RD solution at an interval of 5 min, for the second case. The second layer applied, 4% BG or BG markedly decreased RD permeation in contrast with the second application of aqueous RD solution. Of note, low to no skin permeation of RD was observed with the application of BG as first and second layer.

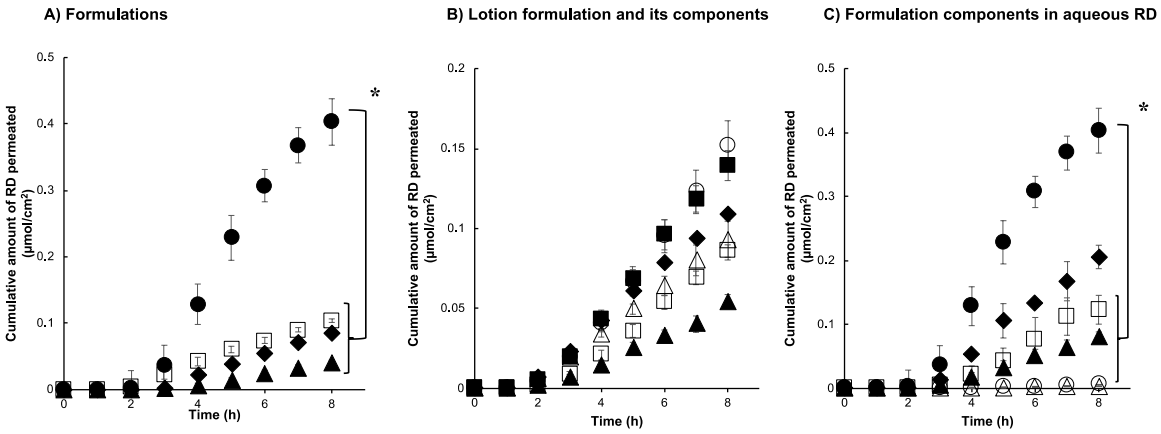

Figure 5. Effect of formulations and formulation components on the skin permeation of RD. a) R-R_{10B} (●), L-L_{10B} (□), M-M_{10B} (♦), $^{(-)}$RD-L_{10B} (▲); b) So-L_{10B} (○), W-L_{10B} (■), 4B-L_{10B} (♦), DP-L_{10B} (Δ), G-L_{10B} (□), $^{(-)}$RD-L_{10B} (▲); c) R-R_{10B} (●), W-R_{10B} (♦), R-$4B_{10B}$ (□), 4B-R_{10A} (▲), BG-R_{10B} (Δ), R-BG_{10B} (○). Each value represents the mean ± S.E. (n = 3-5). *p <0.05. Study code So-L_{10B} refers to layered application of sorbitol as first layer and lotion as second layer with 5 s interval time of application. 4B, BG, G, and DP refers to 4% 1,3-butylene glycol (BG), 100% BG, glycerin, and dipropylene glycol (DPG), respectively. Complete details in Table 3 and section 2.4.

1.3.4. Transepidermal water loss at application site after application of lotion and milk

Figure 6 presents the changes in water loss values after application of lotion or milk. The method directly measured water loss from each time-points as well as detecting total water loss from applied formulations. The application of lotion and milk as the first layer had TEWL values of 0.69 μL/cm^2•h and 0.63 μL/cm^2•h, respectively, at 0.5 min. Water loss at 0-10 min was significantly higher when compared with those prior to application and 10 min onwards for both formulations. The rate of TEWL for applied lotion was significantly faster than milk. A similar controlled monitoring of evaporation was performed by 10 μL water on stacks (4 layers) of filter paper stabilized on a petri dish and was thermostatically maintained at 32 °C. Cumulative amount of water evaporated after 30 mins practically reflects the initial volume applied. Notably, TEWL values for both porcine ear skin and filter paper (data not shown) decreased over time with water loss highest at 0-10 min.

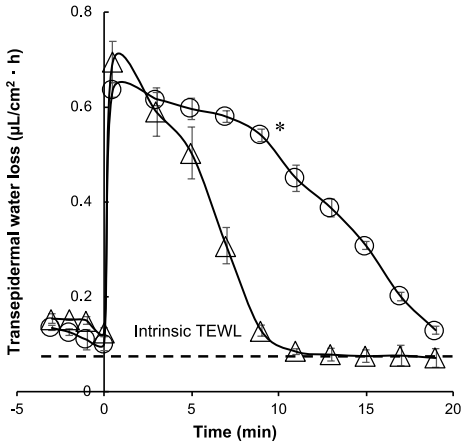

Figure 6. Changes in percent TEWL. TEWL of single application (10 μL/cm^2) of milk (○) and lotion (∆). Each value represents the mean ± S.E. (n = 3-5). Significant difference (*$p < 0.05$) between water loss for milk and lotion at 10 min.

1.3.5. RD Solubility

Table 4 shows the saturated solubility of RD in primary components of the lotion and milk formulations. Consistently, RD had higher solubility with BG along with DPG, and lower solubility with water.

Table 4. Solubility of RD with primary components

Components	Solubility at 30°C (mg/g)
Water	17.28
Aqueous RD, 1%	9.48
BG, 100%	550.16
BG, 5 %	16.549
BG, 10%	18.151
DPG	537.62
Glycerin	117.08
Sorbitol, 70%	3.90
PG	85.34

1.4. Discussion

In the present study, we focused on understanding how cosmetic active components, when applied onto the skin as finite dose would permeate the skin in a similar manner in daily practice, that is applying it in layers of various formulations. Layered application of cosmetics was previously established to reduce the skin permeation of RD, but the mechanism of how "layers" influence the permeation of active components remain poorly understood.

Evaluation of dermal absorption of cosmetic products using skin from mammalian species including humans have long been established; however, due to obvious constraints in availability and ethical concerns associated with the use of human skin, alternatives are widely sought. [50] Edible porcine skin is regarded as being physiologically and morphologically similar [51,52] and is recognized by dermatological scientists to possessing good correlation coefficient (r^2= 0.88), for the permeation of a great number of chemicals, to human skin. [42,48,53,54] Also, the assessment of dermal absorption of cosmetics is ascribed to be performed on porcine skin. [20,22,43,55] Hence,

edible porcine skin was used to evaluate the effect of cosmetics layered application on the skin permeation of RD.

The results in the present study implicated several factors that can influence the permeation of an active component. Since the application volume of 10 µL/cm^2 was sufficient to uniformly cover the effective skin permeation area, the application volumes of 10 and 20 µL/cm^2 were used to elucidate the effect of layered application on the skin permeation of RD. Of note, the application of lotion as the first layer with a longer interval time between applications, 5 and 10 min, exhibited a significant reduction in RD permeation (Fig. 2a). Moreover, short application intervals (5 s) of cosmetics yielded similar results as for permeation after a single application (Fig. 2a, b). On the other hand, permeation data from experiments employing large volumes applied provide evidence suggesting that large volumes (20 µL) of cosmetics applied at the beginning of the permeation experiment resulted in the higher permeation of the active components (Fig. 2a and b).

When layered application was performed with different formulations, changes in the composition of the first and second layer occurred upon mixing at the application site. Physical mixing or addition of other components to formulations comprising oil-in-water or water-in-oil emulsion might cause instability. Even when the same formulation is applied, the composition of the first layer becomes non-identical with the second layer due to the evaporation of solvents (Fig. 3).

In general, elevated skin temperature enhances drug permeation primarily related to increased diffusivity attributed to the increase in the fluidity of stratum corneum lipids leading to increase (expansion) in intercellular space. [56,57] However, the effect of temperature on evaporation and permeation of actives in layered application is not clearly understood. Water loss data revealed that water evaporation

occurred immediately after application of the cosmetics regardless of the type of formulation. High evaporation rates take place within 0- 10 min (Fig 6) suggesting rapid supersaturation of the applied layer and effectively reduced permeation of active component possibly due to crystallization. [58,59] Although polyols such as DPG, BG and glycerin in the formulation are known chemical enhancers for topically applied drugs by increasing their partition coefficient from the formulation into the stratum corneum, permeation of RD after layered application of 10 µL/cm^2 exhibited lower permeation compared with a single application despite having the same application volume (Fig. 4). Moreover, an increase in the concentration of non-volatile components such as DPG, BG and glycerin occurs in the residual phase after the high evaporation rate of water at the time of application. The influence of formulation components on the skin permeation of the active component was confirmed in terms of its effects on solubility and consequential RD permeability. BG (4%) and BG (100%) applied as the first layers resulted to a reduction in RD permeation by 1.6-fold and 80-folds, respectively (Fig. 5). Saturated solubility of RD in BG, DPG and glycerin was much higher compared with water (Table 4). A two-fold increase in RD solubility in water in the presence of 5 – 10% BG and a 58-fold increase with 100% BG was observed. An increase in concentration of polyols is presupposed to promote the solubility of the permeating RD in the residual phase, thereby reducing its thermodynamic activity and consequently the skin permeation of RD. This concept was emphasized by Lane and colleagues describing the importance of high amount of solvent (i.e., water) in the residual phase that should remain on the skin in order to maintain the thermodynamic activity as high as possible to aid in the permeation of the active component. [60] Similarly, the significantly higher solvent evaporation from the lotion could result in an increase in the concentration of RD and/or produce a supersaturated state in formulation

which is a possible mechanism of its increased permeation after a single application and large application volumes (20 µL/cm^2). [61] Differences in the skin permeation of model compounds in previous studies were attributed to changes in drug solubility/ thermodynamic activity in the residual phase induced by the evaporation of solvents (i.e., water) from the formulation. [61,62]

The sequence of application affects the skin permeation wherein milk applied as the first layer resulted in decreased skin permeation of RD (Fig. 4). Lotion, on the other hand, having more water content than milk, produced a less viscous mixture (lower viscosity) rendering RD with a better diffusion environment and subsequently higher permeation extent. [63] Rheological and mechanical properties of formulation are known to affect penetration of actives wherein increase in viscosity results in a reduction in permeation. [64-66]

With a lotion formulation containing no RD applied it as the first layer, it was found that components of lotion formulation altogether resulted in a 2.6-fold decrease in RD permeation (Fig. 5). Furthermore, formulation components were prepared reflective of their respective concentrations in the formulation and applied as the first layers. Formulation components, 4% BG, DPG, and glycerin resulted in 1.3-, 1.5-, and 1.6- fold decrease in RD permeation, respectively (Fig. 5a). In addition, milk formulation contains an exclusive component, PG. PG was previously reported to rapidly permeate, thereby promoting crystallization of the active components further supporting the lower permeation of RD in milk as opposed to lotion. [67] Our findings indicate that to minimize undesired permeation profiles of RD after layered application, lotion should be applied as the first layer with a short interval (<1 min) with respect to its second layer. In addition, it is desirable to have a large initial volume to be applied rather than distantly spaced and applied in divided doses. The design of the

formulations should be reviewed with respect to the active component's solubility and its consequential thermodynamic activity.

As calculated from the 90th percentile of respondents, the amount of lotion used per application, 1.62 g [2] and total facial skin area, 565 cm^2 [21] suggested that approximately 2.8 µL/cm^2 of lotion is the practical amount used per application. Water evaporation rate constant (k_{evap}) was reported to be 2340 $\times 10^{-10}$ ms^{-1}. [68] This would theoretically mean that about 70% of the water in a topically applied formulation at 10 µL/cm^2 would evaporate in 5 min, and about 65% of the water from an applied formulation at 20 µL/cm^{2}s would remain. Thus, in the case of application with doses lower than 10 µL/cm^2, layered application would induce reduction in the skin permeation of RD.

The need for ensuring safety of cosmetic products was recently raised by a group of cosmetic scientists as a large population of women utilize these products on a daily basis over an extended period of time. [2] We have established, for the first time, that layered application and components of a formulation can be investigated with regard to their influence on the skin permeation of actives thus, assessing safety of cosmetics used concurrently or in layers.

1.5. Chapter conclusion

Rapid water loss occurs during the interval time of application between layers. The increase in the concentration of non-volatile polyol components such as DPG, BG, and glycerin in the residual phase promotes the solubility of the permeating RD in the residual phase, thereby reduces its thermodynamic activity and consequently reduces its permeation. Formulations and their components caused varying reductions in RD permeation, probably due to decrease in thermodynamic activity of the active

component. Layered application, formulation components, application volume, time interval and sequence of application had significant influences on the skin permeation of the active component. Layered application of RD-containing cosmetics does not necessarily increase the amount of RD permeating through the skin and this habit of use does not directly cause leukoderma.

Moreover, this study established a method of investigating the influence of formulations and their components on the skin permeation of actives after layered application.

CHAPTER 2

Prediction of skin permeation and concentration of rhododendrol applied as finite dose from complex cosmetic vehicles

2.1. Introduction

A number of cosmetic formulations are made of quasi-drugs (active compounds) effectively dissolved in complex vehicle systems. These formulations may contain components that enhance or decrease the penetration of active compound or other components. In addition, vehicle composition may change after topical application due to low amount of formulation applied. Therefore, the permeation of chemicals from a small amount of topically applied formulation in its in-use conditions is difficult to simulate experimentally. Evaluation of dermal permeation is typically conducted under finite and infinite experiments. The finite dose experiment (non-occluded) is supposed to best represent its clinical use (i.e., in-use conditions) wherein depletion of dose and evaporation of the excipients may occur. On the other hand, an infinite dose experiment (occluded) is characterized by a non-depleting dose. Investigating the percutaneous absorption of chemicals, under its in-use conditions, has been presented with huge challenges associated with incomplete recovery of the applied formulation, low extraction ratio of compounds from the skin, and inter-run variabilities for key parameters (e.g., skin permeability, partition coefficient from vehicle to skin) in such experiments. [19] To date, no definitive method has been established to address these challenges.

The penetration of chemicals from aqueous vehicles in infinite dose models under steady-state conditions (i.e., non-depleting dose) can generally be predicted based

on their physicochemical properties. [40,69] However, steady-state conditions are typically unattainable in finite dose experiments where dose depletion takes place. The assumption of steady-state conditions does not apply to finite dose experiments since a high evaporation rate of applied solvents occurs after application. Generally, rapid evaporation of solvents occurs, which significantly alters the effective diffusion area of the applied formulation and the composition of the resulting residual formulation after formulations are applied on the skin. [23-25] In contrast, the majority of studies done to asses this phenomenon were performed with infinite dose conditions, whereas only a limited number of studies have been conducted for finite dose conditions. Hence, caution must be considered in extrapolating data derived from infinite dose experiments or experiments in which exposure occurs via simple aqueous vehicles, because these do not necessarily reflect the complexities of most formulations used in practice. In addition, few studies have been conducted to predict skin permeation in finite dose settings with the use of actual cosmetic formulations. Appropriate alternatives in modeling this phenomenon must then be adopted to enhance this point and better predict skin permeation for in-use conditions. Predicting skin permeation of cosmetic active compounds in finite dose settings will not only provide insights on local toxicity but also allow prediction of its systemic absorption.

The influence of in-use conditions such as layered application, evaporation in formulations, and sequential and concurrent application of polyols with cosmetic formulation in the skin permeation of cosmetic active compounds has been recently reported. Layered application of cosmetics and concurrent application of polyols dramatically reduced the skin permeation of active compounds. [23] Findings from various reports had diverging claims on the roles of solubility in the skin permeation of chemicals under finite dose conditions. [47,60,70] Several studies have focused on

estimating the amount of chemical permeating through the skin based on the physicochemical properties of permeants and formulations, yet they neglected the essential role of evaporation in the actual permeation of chemicals. [40, 71, 72] Furthermore, little is known about what governs the skin permeation and concentration of chemicals applied as a finite dose.

The efficacy and safety of cosmetics and locally acting drugs applied on skin are determined by their distribution into its intended site of action, most likely the viable epidermis and dermis (VED), and not the stratum corneum (SC). Skin whitening agents from cosmetics or steroids and antimicrobials from topical medications must be studied for their distribution and concentration in the VED. [18] The epidermal layers being the primary site of action for these products offer direct insights for safety assessments or product optimization. The importance of the concentration in the VED is greater for cosmetics and topical medications that are capable of causing skin irritation and inflammation. [73] In recent times, the toxicity of cosmetic active compounds may be represented well by reports on RD– related leukoderma. In this case, evaluation methods for dermatological products based upon appropriate skin models and in-use conditions are important to confirm dose-dependent toxicity of compounds at the site of action. Determining the distribution of chemicals in the VED is of great significance for cosmetic formulations, where they are expected to maintain their effective concentrations. Quantification of permeant concentration in the skin allows a high precision in predicting their efficacies or toxicities.

Establishing mathematical models aimed at predicting skin or VED concentration of chemicals entails understanding of the factors that influence skin permeation. Therefore, this investigation probed the possible role of evaporation and the composition of residual formulations on the skin permeation and concentration in

finite dose conditions. The actual impact of vehicle on the skin permeation and concentration of the penetrant can be realistically clarified by simulating the residual formulation based on evaporation kinetics from applied formulations. We employed various polyols commonly used as solvents in cosmetics and simulated residual formulations composed of high polyol proportions to reveal its role in the skin permeation of active compounds. Here, we propose a method that allows investigation of the permeant disposition from residual formulations encompassing evaporation, which is a natural process during use. This is an extension of our inquiry on the fate of cosmetic active compounds from complex formulations in actual product in-use conditions (e.g., layered application, finite dose conditions). Experiments in steady-state conditions for simulated residual formulations were conducted to allow surrogate estimation of skin permeation parameters in finite dose exposures. In the present study, we attempted to establish a mathematical method in predicting skin permeation and the concentration of cosmetic active compounds applied in finite dose from a complex vehicle-based formulation.

2.2.Materials and methods

2.2.1. Materials

RD (CAS no. 501-96-2, ≥ 99%) was supplied by Kanebo Cosmetics, Inc. (Tokyo, Japan). Methylparaben and glycerin were purchased from Fujifilm Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Sorbitol, trichloroacetic acid and 1,3-butylene glycol (BG) were purchased from Tokyo Chemical Industry, Co. Ltd (Tokyo, Japan) while dipropylene glycol (DPG) was purchased from Sigma Aldrich Chimie (Saint-Quentin-Fallavier, France).

The complex vehicle, a recalled lotion of RD, was supplied by Kanebo Cosmetics, Inc. It was primarily composed of water and a mixture of polyols (DPG, glycerin, BG, and sorbitol; each concentration is shown in 2.2).

2.2.2. Preparation of RD formulations

Aqueous formulation of RD (1%) (Table 1) was prepared by dissolving RD in a sufficient amount of purified water in a volumetric flask. An RD concentration of 1% was selected instead of 2% due to its limited solubility with water.

The polyol mixture was composed of DPG (46.15%), glycerin (23.08%), BG (20.51%), and sorbitol (10.26%) identical to that of the recalled formulation. A prepared lotion formulation (2% RD) containing identical total polyol concentration, 19.5% and water, of the recalled lotion, was also prepared (Table 1).

To reflect formulation conditions in the residual phase, formulations depicting polyol concentration following evaporation were developed. Simulated residual formulations of RD (2%) lotion were designed to reflect varying degrees of evaporation from the formulation hence, polyol concentrations of 40%, 61.8%, and 100% were adopted. These polyol concentrations were particularly selected to reflect low, middle, and high degree of water evaporation in the residual phase. These formulations were prepared by the addition of a sufficient amount of purified water with its corresponding polyol proportions in a volumetric flask.

Table 1. Composition of RD formulations

	Recalled formulation	***Prepared formulations***				
Components (%)	Recalled lotion	1% RD Aqueous	Prepared lotion	2% RD Lotion (40% Polyol)	2% RD Lotion (61.8% Polyol)	2% RD Lotion (100% Polyol)
Rhododendrol	2	1	2	2	2	2
Polyols	19.5	-	19.5	40	61.8	q.s. 100
Water	q.s. 100	q.s. 100	q.s. 100	q.s. 100	q.s. 100	-

2.2.3. In vitro skin permeation experiment

Frozen porcine ears (Central Institute for Feed and Livestock; JA Zen-Noh, Ibaraki, Japan) were thawed with warm water and rinsed with purified water. Hairs were trimmed and shaved, and subcutaneous fats were excised off the skin. Skin was harvested from the central dorsal region of the ears. Before excision, visual inspection was performed to ensure the integrity of the skin. Only intact and damage-free skin was excised. For stripped skin, adhesive tape was applied on the SC side and stripped 20 times prior to excision. Isolated porcine skin was set on vertical type Franz diffusion cells (effective diffusion area of 1.77 cm^2). Skin surface temperature was maintained at 32°C throughout the experiment. The receiver compartment was filled with 6.0 mL of purified water. Prior to the application of doses, the skin was applied with purified water (1.0 mL) to facilitate equilibration for 1 h. Water was then carefully removed and skin surface was blotted with a cotton swab to remove excess water. Using a positive displacement micropipette, RD formulations (1% RD aqueous, 2% RD in 19.5% polyol, 2% RD in 40% polyol, 2% RD in 61.8% polyol, 2% RD in 100% polyol) were applied as either finite (17.7 μL/ 1.77 cm^2) or infinite dose (1.0 mL/1.77 cm^2). At a predetermined schedule, an aliquot (500 μL) was withdrawn from the receiver solution. Permeation experiments were performed for 0 – 4 h or 0 – 8 h.

2.2.4. Skin concentration experiment

The skin concentration of RD was determined using identical experimental conditions as the skin permeation experiment using both intact and stripped skin. Formulations were applied in infinite and finite doses. Skins were demounted from the diffusion cells and adhering formulations were removed at 4 h and 8 h after the start of skin permeation experiment. Skins were rinsed thrice on both sides with purified water

and blotted dry with tissue paper. Tape-stripping (20 times) was performed on the intact skin to isolate the VED. A sample (0.05 g) of the VED was reduced in size using a pair of scissors. Then, water was added and the skin was homogenized using a Polytron PT 1200E (Kinematica, Inc., Luzern, Switzerland) for 5 min. Samples were deproteinized by the addition of 16% trichloroacetic acid. The samples were agitated using a vortex mixer for 15 min, followed by centrifugation (15,000 rpm, 4°C) for 5 min. The supernatant liquid was prepared for quantification.

2.2.5. Water evaporation experiment from formulation

Evaporation of water from the recalled formulation was determined gravimetrically by monitoring weight loss of the applied solvent/formulation over time. The weight of an empty glass-bottom dish was first measured using an analytical balance (AUW220D; Shimadzu, Kyoto, Japan). Balance reading was deemed stable when differing readings are less than ± 0.0001 g within 3 min. A finite dose (17.7 μL) of lotion was evenly applied using a micropipette and the initial weight of the applied formulation was recorded. The set-up was placed on a thermostatically (32°C) maintained heating plate (AS ONE, Osaka, Japan). Surface temperature was monitored (32 ± 1°C) using probe and infrared thermometers throughout the experiment. Water loss (weight of the setup) was recorded over time at intervals of 1 min until constant weight was attained.

2.2.6. Determination of solubility of RD in residual formulations

The solubility of RD was performed in a wide range of polyol concentrations (10.0%, 19.5%, 40.0%, 61.8%, and 100%) simulating various stages of evaporation in the residual formulations based on a previous work [5]. The excess amount of RD was

stirred inside a capped vial immersed in a thermostatically controlled water bath (32°C) for 48 h. This approximated the solubility of RD in the residual formulation on skin. Dissolved RD in solvents/simulated residual formulations were filtered using a polytetrafluoroethylene membrane syringe filter (Advantec®, 0.2 µm, Tokyo, Japan) and analyzed using HPLC.

2.2.7. HPLC analysis

RD was analyzed using HPLC as described previously. [23] Briefly, 100 µL of samples were added with an equal amount of internal standard (methylparaben) and centrifuged at 4°C for 5 min. Samples were injected into an HPLC system and analyzed for RD concentration at 280 nm.

2.2.8. Theoretical

2.2.8.1.Concentration-distance profile of a penetrant in SC and VED

Skin diffusion model of a penetrant is generally expressed in its concentration-distance profile as shown in Figure 1. As such, a two-layered diffusion model can be used for penetrant diffusion through the full-thickness skin (SC + VED double membrane) while one-layered diffusion model is sufficient for SC-stripped skin (VED single membrane).

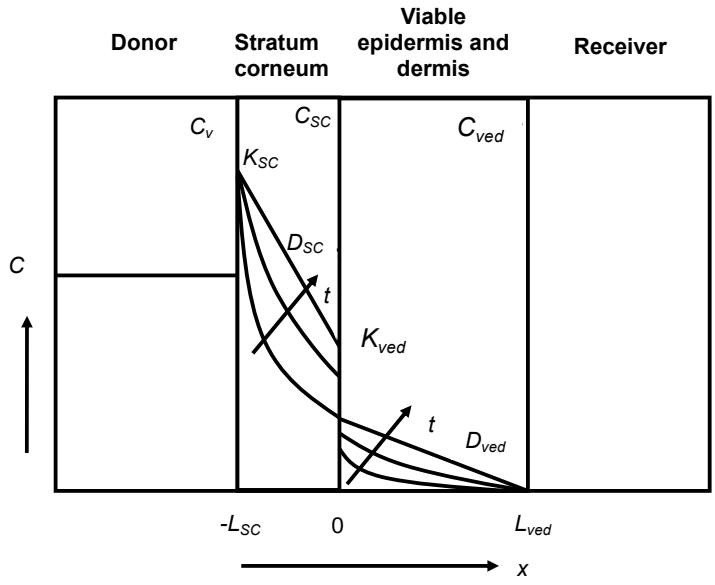

Figure 1. General concentration-distance profile of a penetrant in two-layered membrane diffusion model. C_v, C_{sc}, C_{ved} refers to the penetrant concentration in the vehicle, SC and VED, respectively; K_{sc} and K_{ved} refer to partition coefficients from the donor (vehicle) to SC and VED, respectively; D_{sc} and D_{ved} are diffusion coefficients in the SC and VED, respectively. L_{sc} and L_{ved} refer to thicknesses of the SC and VED, respectively; and t refers to time after starting the permeation experiment.

2.2.8.2.Fick's second law of diffusion and related initial and boundary conditions

In the case of a two-layered diffusion model under infinite dose condition, SC and VED concentration of a penetrant (C_{sc}, and C_{ved}) at position, x, and time, t, can be described by the following Fick's second law of diffusion described in our previous papers. [18, 73-75]

$$\frac{\partial C_{sc}}{\partial t} = D_{sc}\frac{\partial^2 C_{sc}}{\partial x^2} \quad (1)$$

$$\frac{\partial C_{ved}}{\partial t} = D_{ved}\frac{\partial^2 C_{ved}}{\partial x^2} \quad (2)$$

where D_{sc} and D_{ved} are the effective diffusion coefficients of a penetrant in SC and VED, respectively.

Initial and boundary conditions for penetrant concentration in infinite dose system were as follows:

$$t=0 \qquad -L_{sc} < x < 0 \qquad C_{sc} = 0 \qquad (3)$$

$$0 < x < L_{ved} \qquad C_{ved} = 0$$

$$t>0 \qquad x = -L_{sc} \qquad C_{sc} = K_{sc} \cdot C_v \qquad (4)$$

$$x = 0 \qquad C_{ved} = K_{ved} \cdot C_{sc} \quad \text{and}$$

$$D_{sc} \frac{dC_{sc}}{dx} = D_{ved} \frac{dC_{ved}}{dx}$$

$$x = L_{ved} \qquad C_{ved} = 0$$

where L_{sc} and L_{ved} are the thicknesses of SC and VED, respectively; K_{sc} and K_{ved} are the partition coefficients of the penetrant from the donor (vehicle) to SC and VED, respectively; C_v is penetrant concentration in the applied formulation (donor or vehicle). In the present RD permeation experiments through excised porcine ear skin, L_{sc} and L_{ved} were set to be 20 μm and 1480 μm, respectively.

Against Eq. (4) for the infinite dose system, the boundary condition only at x = 0 in the finite dose system becomes,

$$t > 0 \qquad x = -L_{sc} \quad C_{sc} = K_{sc} \cdot C_v \qquad (4')$$

$$x = 0 \qquad V_v \frac{dC_v}{dt} = A\, D_{sc} \frac{dC_{sc}}{dx}$$

$$x = L_{ved} \qquad C_{ved} = 0$$

where V_v is the volume of the vehicle (donor solution); A is the effective permeation area. The equation in the second line in Eq. (4') means that the decrease in flux of the penetrant in the donor compartment is equal to the increase in flux at x = 0 in the SC.

When the amount of the penetrant permeated in the finite dose through membrane is very low, Eq. (4) can be used instead of Eq. (4'). Only a small amount of RD permeated through the skin in the case of the present RD skin permeation experiment, suggesting that Eq. (4) can be used for Eq. (4') even at finite dose.

2.2.8.3.Equations to determine the skin permeation rate and amount of a penetrant

The skin permeation rate of penetrant per unit area, *J*, is expressed by Eq. (5) using Fick's first law of diffusion. The cumulative amount of the penetrant permeated per unit area, *Q*, is determined by integrating Eq. (5). *Q* is expressed by Eq. (6).

$$J = -D_{ved}\left(\frac{dC_{ved}}{dx}\right)_{x=L_{ved}} \quad (5)$$

$$Q = -D_{ved}\int_0^t \left(\frac{dC_{ved}}{dx}\right)_{x=L_{ved}} dt \quad (6)$$

These equations can be applied to both the infinite and finite dose systems.

2.2.8.4.Determination of D_{ved}, D_{sc}, K_{ved} and K_{sc}

The K_{ved} and D_{ved} can be obtained from permeation experiment using SC-stripped skin (VED single membrane) in the infinite dose system [74] (Details are shown in 2.2.8.6). Then, K_{sc} and D_{sc} are determined by the permeation experiment using full-thickness skin (SC + VED double membrane) in the infinite dose system. The obtained K_{ved} and D_{ved} values were fixed for calculating K_{sc} and D_{sc}.

2.2.8.5.Determination of $C_v(t)$ and $K_{sc}(t)$

RD formulations consisted of water and polyol mixture (Table 1) were applied on skin in the present study. Water evaporated from the formulation whereas polyols remained on the skin in the present finite dose experiments. Thus, C_v and K_{sc} must be expressed as a function of time as in $C_v(t)$ and $K_{sc}(t)$. Then, the C_v of RD in different concentrations of polyol vehicles (19.5%, 40%, 61.8% and 100%) was determined, and in each concentration of polyols, C_v was calculated using spline interpolation. Time course of the polyol concentration was determined by the water evaporation data from formulation (see 2.2.5 in detail). Finally, the time course of $C_v(t)$ was obtained.

In addition, K_{sc} of RD from vehicles composed of 19.5, 40, 61.8 and 100% polyols were experimentally determined by the permeation experiment through full-thickness skin using infinite dose conditions. Permeation experiments through stripped skin were also done as mentioned above. The K_{sc} of RD from each concentration of polyols in the formulation to SC was then calculated using spline interpolation. The time course of the polyol concentration was determined by the water evaporation data as above. Thus, the time course of $K_{sc}(t)$ was obtained as like in $C_v(t)$.

2.2.8.6.*Differential equations to obtain C_{sc} and C_{ved} at any time and any position*

Differential equations describing Fick's second law of diffusion are as follows:

$$\frac{dC_{i,j}}{dt} = \frac{1}{\Delta t}\left(C_{i,j+1} - C_{i,j}\right) \quad (7)$$

$$\frac{d^2C_{i,j}}{dx^2} = \frac{1}{\Delta x^2}\left(C_{i-1,j} - 2C_{i,j} + C_{i+1,j}\right) \quad (8)$$

where $C_{i,j}$ shows concentration of penetrant in SC or VED at an i-th skin position and a j-th time after starting the skin permeation experiment (both i and j are natural numbers), and Δx is $x_{i+1} - x_i$ and Δt is $t_{j+1} - t_j$. Fick's second law of diffusion (Eqs. (1)

and (2)) is expressed using the following differential equations, Eqs. (7) and (8). The following, Eq. (9), was obtained from Eqs. (7) and (8).

$$C_{i,j+1} = rDC_{i-1,j} + (1 - 2rD)C_{i,j} + rDC_{i+,j} \quad (9)$$

where $r = \Delta t/\Delta x^2$. Eqs. (5) and (6) can be expressed using these differential equations as follows:

$$J_j = -D_{ved}\frac{C_{n+1,j} - C_{n,j}}{\Delta x} \quad (10)$$

$$Q_j = Q_{j-1} + J_j \Delta t \quad (11)$$

where *n* is the number of divisions of SC or VED.

2.2.8.7.Determination of J_j and Q_j

J_j and Q_j were calculated using a spreadsheet, Microsoft® Excel by setting n = 10 both for SC and VED. In this calculation, Δt was set to be less than 0.5 for $D_{sc}\cdot r$ or $D_{ved}\cdot r$, because the solution will diverse at 0.5 or more for $D_{sc}\cdot r$ or $D_{ved}\cdot r$. Q_j was calculated from J_j using Eq. (11). First, experimentally observed Q values (Q_j) at every sampling time point in the infinite dose system were fitted by the least-squares method calculated using a quasi-Newtonian method in Microsoft® Excel Solver. [76] Permeation parameters such as partition coefficients K_{sc}, K_{ved}, diffusion coefficients D_{sc}, D_{ved}, and permeability coefficient (K_p) were calculated using the analytical method described in our previous work. [74]

C_{sc}, at any t (at $x = -L_{sc}$), $C_{sc}(t)$ at $x = -L_{sc}$, was calculated by the following equation:

$$C_{sc}(t) = K_{sc}(t) \cdot C_V(t) \qquad (\text{at } x = -L_{sc}) \quad (12)$$

where $K_{sc}(t)$ and $C_v(t)$ are obtained as shown in 2.2.8.5. We inputted $C_{sc}(t)$ (at $x = -L_{sc}$) in the spreadsheet in the present calculation. This was a kind of sequential approach to derive the calculation method.

2.2.8.8.Diagram of calculation method for C_{sc} and C_{ved}

In this work, permeation parameters, K_{sc}, K_{ved}, D_{sc} and D_{ved}, from 1% RD aqueous solution through intact and stripped skin were initially determined in the infinite dose system. Figure 2 presents a detailed flow diagram to obtain $K_{sc}(t)$and $C_v(t)$.

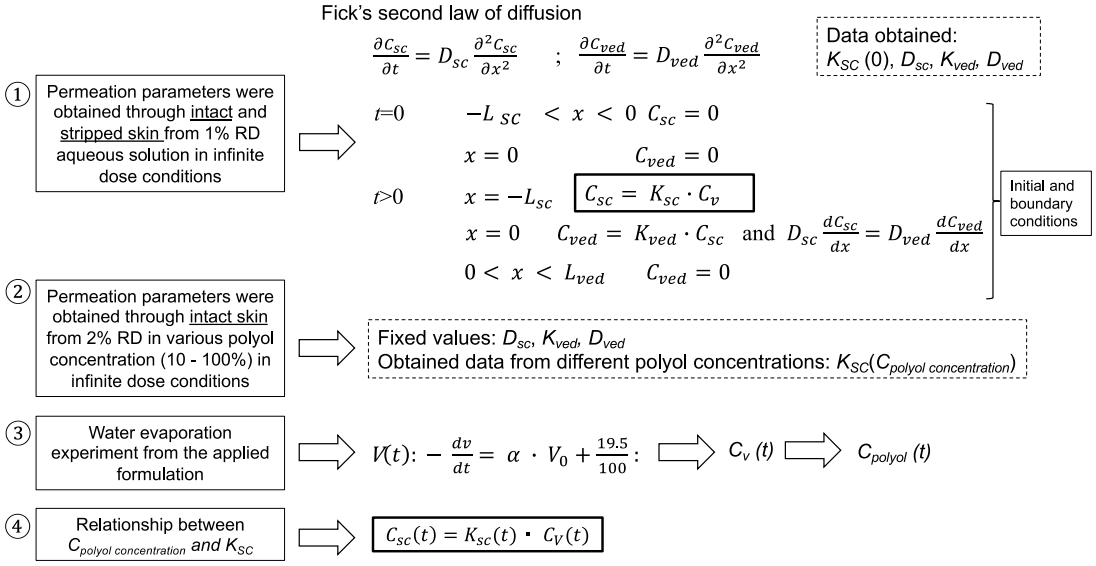

Figure 2. Flow diagram for time course of $K_{sc}(t)$ and $C_v(t)$. C_v, C_{sc}, C_{ved} refers to the penetrant concentration in the vehicle, SC and VED, respectively; K_{sc} and K_{ved} refer to partition coefficients to SC and VED, respectively; D_{sc} and D_{ved} are diffusion coefficients in the SC and VED, respectively. L_{sc} and L_{ved} refer to thicknesses of the SC and VED, respectively; t refers to time after starting the permeation experiment.

2.3 Results

2.3.1 Evaporation of water from applied formulation

Water evaporation was evaluated from a recalled lotion formulation of RD solubilized in a complex mixture of polyols (DPG, glycerin, BG, and sorbitol).

Evaporation kinetics from the applied formulation was measured gravimetrically. The use of a glass-bottom dish allowed accurate measurement of water evaporation from the formulation applied as opposed to the use of isolated skin where intrinsic water loss may lead to overestimation. Observed data for water evaporation was in agreement with previous study [23] where ~60% of total water content evaporated within the first 10 min (Fig. 3). The amount of water detected (96.3%) at the end of the experiment corresponded closely to the actual water content of the recalled formulation. Exhaustive evaporation of water from the applied formulation was observed in this study. The evaporation rate from the formulation exhibited first-order kinetics and the percent water loss, *y*, was calculated using the following equation, $y = 103 \times e^{-0.093t}$, where *t* is the time after the start of experiment (Fig. 3).

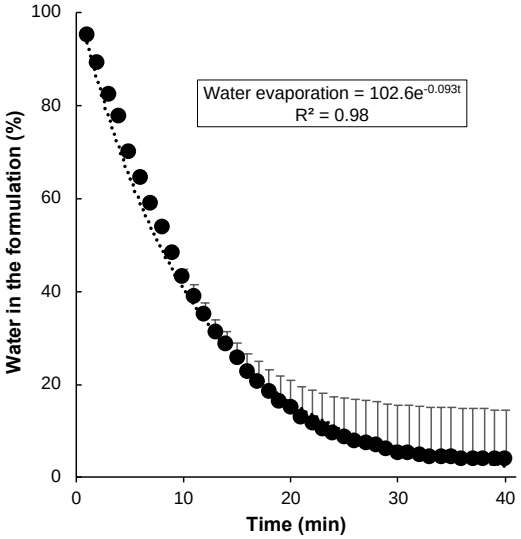

Figure 3. Percent water loss from applied formulation. Each point represents the mean ± S.D. (n=4).

2.3.2 Skin permeation of RD from aqueous formulation

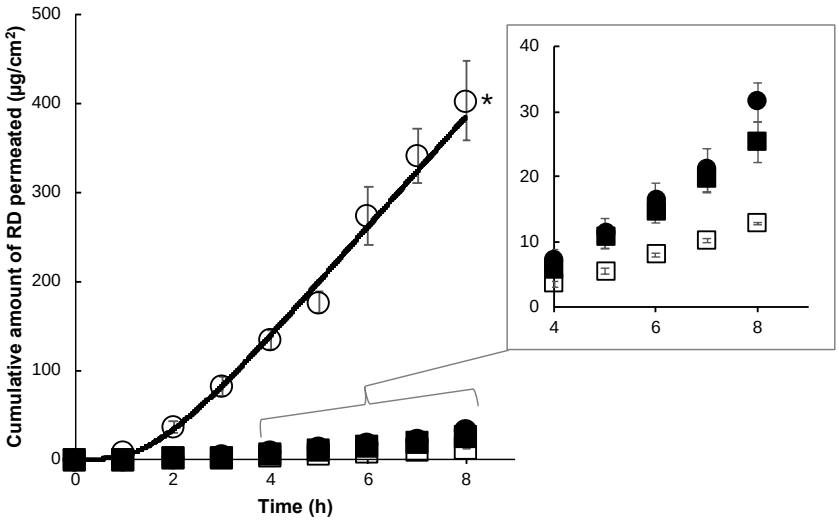

Figure 4. Time course of the cumulative amount of RD permeated through skin under infinite dose conditions. Permeation profiles from 1% Aqueous RD through intact skin (●); 1% Aqueous RD through stripped skin (○); 2% RD lotion through intact skin (■); and 2%RD lotion through stripped skin (□); line represents the predicted profiles of RD. Each point represents the mean ± S.E. (n=4). Significant difference ($^{*}p$<0.05) between 1% Aqueous RD and 2% RD in 19.5% polyol through stripped skin.

Figure 4 presents the time course of the cumulative amount of RD permeated through intact and stripped skin. RD permeation was 13-fold higher through stripped skin from 1% RD aqueous solution compared with intact skin. Permeation parameters, diffusion coefficients (D_{sc}, D_{ved}) and partition coefficients (K_{sc}, K_{ved}) were obtained by curve-fitting the cumulative amounts of RD that permeated through intact and stripped skin to the theoretical values using a least-squares method. Table 2 shows the calculated values of the permeation parameters.

Table 2. Permeation parameters from RD formulations in various polyol concentration

	Formulations	
Parameters	1% RD Aqueous	Recalled lotion
D_{sc} *(cm²/h)*	9.0×10^{-6}	4.6×10^{-6}
D_{ved}*(cm²/h)*	1.8×10^{-3}	1.8×10^{-3}
K_{sc}	0.50	0.135
K_{ved}	0.56	0.56

2.3.3 Skin permeation profiles of RD from infinite dose experiments

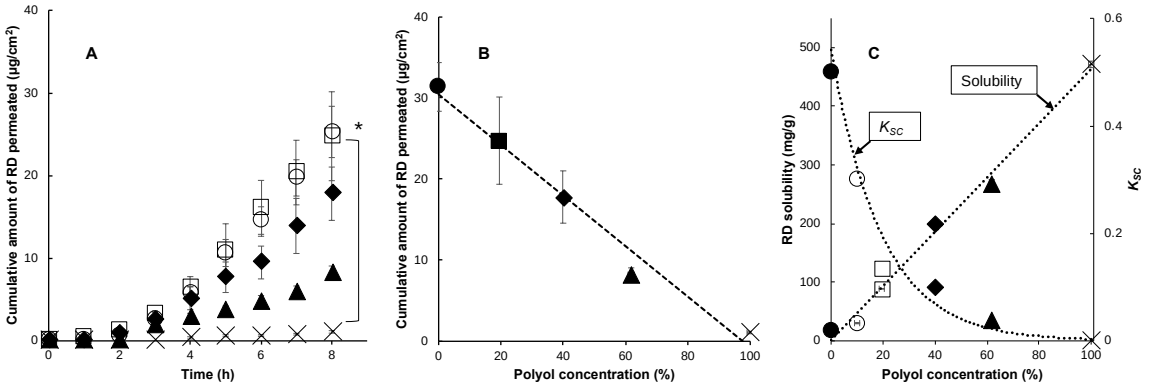

Figure 5. Relationship of polyol concentration, RD solubility and permeation. (A) Cumulative RD permeation from lotion formulations through intact skin under infinite conditions; Prepared lotion (○); Recalled lotion (□); 2% RD in 40% polyol (◆); 2% RD in 61.8% polyol (▲); 2% RD in 100% polyol (×). Significant difference (*p<0.05) between 2% RD in 100% polyol and 1% Aqueous RD, 2% RD in 19.5% polyol, or 2% RD in 40% polyol. (B) Relationship between polyol concentration and cumulative amount of RD permeated at 8 h. 1% Aqueous RD (●); 2% RD in 19.5% polyol (■); 2% RD in 40% polyol (◆); 2% RD in 61.8% polyol (▲); 2% RD in 100% polyol (×). (C) Relationship between polyol concentration with RD solubility and partition coefficient. 1% Aqueous RD (●); 2% RD in 10% polyol (○); 2% RD in 19.5% polyol (□); 2% RD in 40% polyol (◆); 2% RD in 61.8% polyol (▲); 2% RD in 100% polyol (×) Each point represents the mean ± S.E. (n=4).

Figure 5A shows the cumulative amount of RD permeated through intact skin from lotion with different polyol concentrations (19.5% - 100%). Recalled lotion and prepared lotions, having identical proportions (19.5%) of polyols, resulted in similar skin permeation profiles with negligible variances. The K_{ved}, D_{sc}, D_{ved} values obtained from skin permeation experiments using 1% RD aqueous solution were fixed to estimate K_{sc} of RD formulations with varying polyol concentrations (Table 3). Formulations with high polyol concentrations resulted in low K_{sc} values.

2.3.4 Relationship between polyol concentration and RD permeation

Figure 5B presents the correlation between the cumulative amount of RD that permeated through porcine skin and polyol concentration. When the polyol

concentration increased from 19.5 to 40%, 61.8 and 100%, the skin permeation of RD was reduced by 1.8-, 3.8-, and 28.8-fold, respectively. The skin permeation of RD exhibited a positive inverse correlation (r^2 = 0.98) against the polyol concentration in formulation, suggesting that a high polyol concentration would yield lower skin permeation of RD.

2.3.5 Solubility of RD in simulated residual formulations

Solubility of RD in the simulated residual formulations revealed a positive linear correlation (r^2 = 0.99) with the polyol concentration in the formulations (Fig. 5C). High solubility of RD was observed in residual formulations containing high polyol concentrations (90.44 to 100%) and likewise low solubility at lower polyol concentrations (19.5 – 40%) (Fig. 5C). In residual formulation containing 61.8% polyols, wherein its water concentration was about half of its original concentration in the recalled lotion, yielded a 3-fold increase in RD solubility.

Water evaporation from formulation increased polyol concentration in the residual phase induced changes in the K_{sc} (Table 3). A high polyol concentration in the formulation was correlated with lower K_{sc} values (r^2 = 0.96; $K_{sc} = 0.54e^{-0.052x}$, where x is the concentration of polyol in the formulation).

2.3.6 Prediction of skin permeation and concentration of RD from complex cosmetic formulations

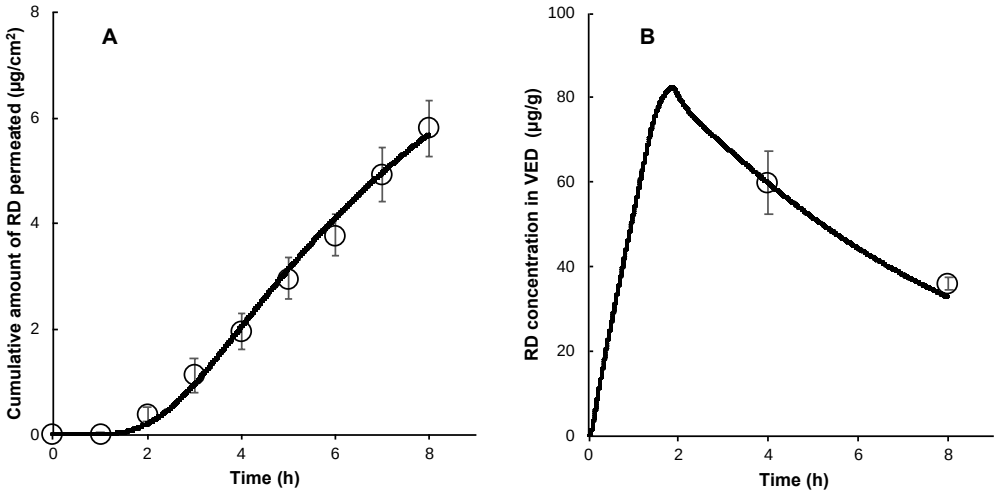

Figure 6. Time course of the cumulative amount permeated through skin (A) and concentration in VED (B) of RD recalled lotion under finite dose conditions. Unfilled circles (○) represent experimental data while lines represent the predicted profiles of RD. Each point represents the mean ± S.E. (n=4).

Figure 6 presents the time course of the cumulative amount of RD that permeated through skin and the concentration in the VED from recalled lotion. C_v was obtained from water evaporation in the formulations and the decrease in the amount of RD in the formulation by permeation through skin over time. The actual experimental data were plotted against the predicted values and well-fitting lines were observed in both skin permeation and concentration.

Table 3. Calculated permeation parameters of RD through intact porcine skin

	Polyol concentration (%)				
Parameters	0	19.5	40	61.8	100
J (µg/cm²/h)	8.31 ± 1.17	4.60 ± 0.74	3.37 ± 0.59	1.51± 0.12	0.21 ± 0.03
P (cm/s)	(6.55 ± 1.75) x 10^{-07}	(7.25 ± 1.16) x 10^{-07}	(4.09 ± 0.72) x 10^{-08}	(1.41 ± 0.13) x 10^{-08}	(1.56 ± 0.23) x 10^{-09}
K_{sc}	0.5	0.14	0.10	0.04	0.002

2.4 Discussion

In the present study, we assumed that RD solubilized in complex polyol vehicles penetrate the shallow segment of the SC. Hence, K_{ved}, D_{sc}, D_{ved} were fixed and used in estimating K_{sc} of RD solubilized in polyol vehicles. This phenomenon is mainly

influenced by two factors; high polyol concentration and water evaporation from formulation on the skin surface. These factors alter the drug partitioning into the SC and consequently regulate the amount of the permeants in and through the skin.

Evaporation of volatile components from applied formulations occurs particularly in finite dose conditions and clinical applications. This highlights the fact that the actual permeation of chemicals through skin is best manifested by simulating the conditions of the residual formulations wherein complete evaporation occurs in the residual phase. The rate of evaporation in the residual phase of the formulation determines its effective area of diffusion. The increase in polyol concentration in the residual phase caused by water evaporation is thus a major determinant in the skin permeation of active compounds. By using a broad range of polyol concentrations in simulating the residual formulations, a mechanistic approach can be provided to investigate the impact of evaporation in the skin disposition of RD.

Permeation of RD through intact and stripped porcine ear skin under infinite dose conditions was determined to evaluate the partition and diffusion parameters of RD. The well-fitting line was obtained for RD allowing estimation of the effective diffusion coefficient in the VED by considering evaporation kinetics (Fig. 3) and the related changes in the C_v and K_{sc} (Fig. 5C). The same observation was reported by Potts and Guy in predicting the permeability of chemicals through skin from aqueous solutions. [71] However, this was not observed in the case of a finite dose since the predicted parameters yielded poor-fitting line and thus, imprecise estimation of RD concentration in the VED (data not shown).

For infinite dose conditions, the formulation dynamics are maintained throughout the experiment with the concentration gradient favoring passive diffusion,

a condition obeying Fick's first law. However, in a finite dose setup, the permeation environment is abruptly altered after application of the formulation. This 'new' environment, residual formulation, therefore dictates how chemicals permeate through the skin in finite dose exposures. Otto et al. stressed the need to understand the impact of evaporation on the formulations and the consequent transformations it undergoes after application onto the skin taking into consideration that the actual permeation occurs after complete evaporation from the residual formulation. [24] This is a factor largely ignored despite the fact that the residual formulation differs considerably from the original formulation prior to application. [25] In the present study, the prediction of skin permeation and VED distribution was greatly improved upon incorporating evaporation rates of concerned formulations. A goodness-of-fit was observed for the RD permeation through porcine skin from the lotion formulation (Fig. 6).

Generally, evaporation from the residual formulation affects the permeation parameters. These parameters are determined by the interactions between the permeant and the formulation. It was clear in the present study that water evaporation altered the solubility of RD in the residual polyol vehicles. The effect of water evaporation on the skin permeation of RD from residual formulations was confirmed in infinite dose experiments, where a decrease in the skin permeation of RD was noted with an increase in polyol concentration in the vehicles (Fig. 5A and B). Estimating the skin permeation parameters from a residual formulation applied as a finite dose has not been realistically achieved because formulations tend to evaporate completely leading to incomplete recovery of formulation for quantification. By simulating the composition of a residual formulation and performing permeation experiments under infinite dose conditions, it mimics permeation in the residual phase and allows a reliable estimation of parameters.

Ideally, to increase the penetration of chemicals into the SC, the solubility of the permeating chemical with the SC must be enhanced by increasing its partition coefficient, K_{sc} or by reducing its solubility in the vehicle. [77] In the present study, we found that the solubility of RD in the residual formulation, with very high polyol concentration, proportionally reduced K_{sc} through solvent evaporation. RD formulations in polyol had significantly lower K_{sc} values relative to the aqueous formulation (Fig. 5C). RD permeation decreased when the amount of polyol (19.5% versus polyol concentrations ≥ 40%) in the residual formulation was more than the required amount to dissolve RD. Permeation and consequential distribution of RD in the VED appeared to be closely related to the thermodynamic activity of RD in the vehicle, as manifested by its partition coefficient.

In the steady-state experiments, RD fluxes of simulated formulations with high polyol concentrations were shown to be reduced as the polyol concentration was increased. An inverse relationship existed between the flux and high polyol concentration (Table 3). Calculated permeation parameters further revealed that an increase in polyol concentration decreased flux and the permeability coefficient. The decrease in partition coefficient for RD in the residual formulation supported the observed lower permeability coefficient in relation to polyol concentration leading to a reduction in flux and consequently RD permeation.

The unintended retention of RD on the skin surface instead of benefiting from a ‘solvent drag effect’ with the use of polyols, typically employed in cosmetics as solvents, can be explained by its solubility in the residual formulation. In this experiment, RD was found to be highly soluble with specific polyols (e.g., BG, DPG) as well as increasing solubility with higher polyol concentration in the residual formulation. Enhancement of solubility of the permeating active compounds in the

formulation (i.e., residual formulation) more than that in the SC resulted in reduced partitioning into the skin. The increase in its solubility will reduce its thermodynamic activity, thereby creating a weaker driving force for diffusion. Complete evaporation of volatile components from the formulation means the chemical in the residual phase has the same thermodynamic activity as in the simulated residual formulation composed of 100% polyol. [78] In fact, the polyols involved in this investigation possess similar polarities and thus lack the ability to limit the solubility of RD. Further evidence on the influence of formulation polarity is the low permeability of RD through stripped skin (Fig. 4). Lotion formulation is presumably lipophilic and the absence of the lipophilic barrier, SC, generates a non-ideal diffusion interaction with the hydrophilic VED. These conditions had unfavorable effects on the formulation where the relative activity coefficient of RD was reduced and partitioning into the skin was hampered. [79] Hence, RD penetrates poorly.

Of note, unrealistic similarities in the skin permeation of RD were observed in infinite dose experiments for aqueous and prepared lotion (19.5% polyol) (Fig. 4). This affirmed the effect of steady-state conditions in possibly overestimating key parameters. The extent of impact of solvent evaporation and enhanced polyol concentration on RD disposition, however, were revealed in data obtained from residual formulations with high polyol concentrations (40%, 61.8%, 100%) where significantly lower values were observed. For formulators, seemingly acceptable permeation may be observed with formulations containing already desirable proportions of polyols (i.e., 19.5%) although permeation of active compounds from the residual formulation is indeed in its altered (evaporated) state.

Although the present study established a practical approach in estimating skin permeation and the concentration of RD from complex vehicles under finite dose

conditions, other factors such as saturated formulations, other types of formulations, solvents, and cosmetic excipients that may affect skin permeation and concentration must be investigated further. The assumption in this study is applicable to a two-layered model where active compounds predominantly permeate through the SC. Hence, the contribution of the hair follicle pathway in the permeation of hydrophilic active compounds must be recognized in future studies.

2.5 Chapter conclusion

In conclusion, we investigated the skin permeation and concentration of an active compound in cosmetics, RD, from a complex vehicle as how it would perform in finite dose exposure. Incorporating evaporation kinetics and vehicle-permeant dependent parameters (K_{sc}, K_{ved}, D_{sc}, D_{ved}) may dramatically improve the precision of mathematical models in predicting the permeation and distribution of active compounds in the skin. Predicting these parameters from a complex vehicle made up of actual cosmetic solvents was previously unattainable due to the fact that steady states were not possible in finite dose models. The use of residual formulations simulating the conditions (changes in polyol composition) of the applied finite dose under infinite experiment conditions have paved the way in the calculation of parameters and significantly enhanced estimation of permeant disposition. Safety assessments of permeating chemicals from complex vehicles in clinical and finite dose exposures may now be sufficiently evaluated for their distribution in the VED using simulated residual formulations.

CHAPTER 3

Usefulness of artificial membrane, Strat-M®, in the assessment of drug permeation from complex vehicles under finite dose conditions

3.1. Introduction

Prohibitions on the use of animals in testing finished cosmetic products have been in effect since 2004. With the amendment in regulatory policy, the applications of *in vitro* methods without the use of animal tissues have gained significant attention as tools for the assessment of skin permeation of cosmetic ingredients. Replacements for skin membranes involving the use of artificial membranes (e.g., Strat-M®, silicone membrane) designed to mimic human and animal skin offer a competent alternative to estimate permeation of drugs through skin. [18, 33, 34, 51, 80]

Strat-M® is an artificial membrane envisioned as an alternative to animal skin. It was engineered to mimic key structural and chemical features of human skin. This multi-layer artificial membrane possesses a tight top layer coated with a lipid blend resembling the lipid chemistry of the human stratum corneum (SC) and a porous lower layer resembling epidermis and dermis layers. [36] This membrane possesses equivalency to human skin for the skin permeation of many drugs and claims to have better correlations compared with other biological membranes. [37] In 2015, a study reported an assessment of the permeation of several chemicals solubilized in phosphate-buffered saline (PBS, pH 7.4) through Strat-M® under infinite dose models. [40] A good correlation coefficient for the permeation of chemicals was found between the artificial membrane with rat and human skin. Recently, a study on the permeation of nicotine from formulations with binary solvents (water and chemical penetration enhancers) applied as a large finite dose (200 μL/0.64 cm^2) reported similar findings, where a good

correlation between the artificial membrane and human skin was found. [36] Other advantages associated with the membrane are its simplicity of handling, low-cost, and low variability of lot-to-lot quality as opposed to animal-based models.

Cosmetic formulations usually lack additives (e.g., chemical penetration enhancers) that alter skin barrier function and promote percutaneous absorption of low molecular weight ingredients. This positions Strat-M® as a suitable substitute for membranes of biological origin in the assessment of 'simple' topical formulations, such as cosmetics. Vehicles used in cosmetics are mostly composed of complex mixtures containing components other than water. Therefore, the effect of the combination of components on the permeation through artificial membranes must be investigated. The use of Strat-M® as an alternative membrane in cosmetics for development, product optimization, regulatory compliance, and safety assessments has been encouraged by several reports. However, the actual suitability of this material has not yet been verified in the context of the in-use conditions of cosmetics. [36-40] Merck Millipore provides a limited list of pure solvents and binary vehicles deemed compatible for use with Strat-M® in the assessment of chemical permeation, but there is a lack of data on polyols, which are common ingredients in cosmetic formulations. [37]

The usefulness of the Strat-M® artificial membrane in the assessment of the permeation of cosmetic active ingredients from complex formulations in in-use conditions has not been clarified. No published data were found on the comparative performance of Strat-M® regarding the permeation of cosmetic active ingredients through porcine skin, despite the recommendations made. Additionally, the recommended use of porcine skin in the assessment of the permeation of cosmetic active ingredients has not been changed. Assessment of the dermal absorption of

cosmetic active ingredients is recommended to be performed using porcine skin, because it resembles closely human skin properties, such as permeability to chemicals, thickness and lipid composition. [41] Being the membrane of choice, it is imperative to understand the similarities and establish equivalency and the relationship between Strat-M® and porcine skin in terms of membrane characteristics (e.g., permeability coefficient, flux, permeation of penetrants) to confirm its applicability in evaluating the permeation of cosmetic active ingredients. The appropriate conduct of *in vitro* skin permeation studies must encompass dose, and the vehicle/formulation should represent the in-use conditions of the intended cosmetic product. The Scientific Committee in Consumer Safety (SCCS) stipulates the conditions for *in vitro* dermal absorption studies of cosmetics, where experimental dose or the amount applied during use (i.e., layered application), formulation (e.g., finished cosmetics products, complex vehicles), and barrier integrity must be met. Furthermore, sample application during *in vitro* experiments should mimic human exposure normally at 10 $\mu L/cm^2$ for liquid formulations. [19, 20, 23, 55] In this study, the design of permeation experiments encompassed finite and infinite dose conditions, layered application, the effect of solvents/complex vehicles, and residual formulations to establish the usefulness of the artificial membrane in assessing the permeation of RD, a lipophilic molecule, and CF, a hydrophilic molecule, as model drugs in simulated in-use conditions. Usefulness and membrane-permeation characteristics were evaluated by comparing these parameters with porcine skin.

3.2. Materials and methods

3.2.1. Materials

Rhododendrol [(RD), (CAS no. 501-96-2, ≥ 99%)] was a gift from Kanebo Cosmetics, Inc. (Tokyo, Japan). Caffeine (CF), methylparaben, and glycerin were purchased from Fujifilm Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Sorbitol and 1,3 – butylene glycol (BG) were purchased from Tokyo Chemical Industry, Co. Ltd (Tokyo, Japan) while dipropylene glycol (DPG) was purchased from Sigma Aldrich Chimie (Saint-Quentin-Fallavier, France). Strat-M® was purchased from Merck Millipore (Tullagreen, Carrigtwohill, Ireland). Frozen porcine ears were supplied by the Central Institute for Feed and Livestock (JA Zen-Noh, Ibaraki, Japan).

3.2.2. Preparation of formulations

Aqueous formulations of CF and RD (1%) were prepared by dissolving a sufficient amount of the drug with purified water in a volumetric flask. The concentration of 1% for RD in water was selected instead of 2% due to its limited solubility.

For complex vehicle-based formulations, a polyol stock composed of DPG (46.15%), glycerin (23.08%), BG (20.51%), and sorbitol (10.26%) was first prepared. CF (1%) formulations with high polyol proportion (50 and 75%) and a simulated residual formulation composed of 100% polyol were derived from the stock. RD (2%) formulations with low polyol proportion (19.5%), high polyol proportions (40 and 61.8%), residual formulations (90.4 and 100%) were also prepared.

3.2.3. In vitro skin permeation experiment

Porcine skin was isolated from frozen edible porcine ears. The preparation and isolation of porcine ear skin was performed according to a previous report. [23] To ensure uniformity, skin from the central dorsal region of the ears was harvested. Before

excision, visual inspection was performed to ensure the integrity of the skin. Only intact and damage-free skin was excised. Isolated porcine skin was set in a vertical-type Franz diffusion cell (effective diffusion area of 1.77 cm^2). Skin surface temperature throughout the experiment was maintained at 32°C. The receiver compartment was filled with 6.0 mL of purified water. Prior to the application of doses, the skin was hydrated with purified water (1 mL) for one hour. Water was then carefully removed and skin surface was blotted with a cotton swab to remove excess water. For Strat-M® experiment, the membrane was directly set in to a vertical-type Franz diffusion cell with the polyether sulfone side (shiny top layer) upwards. Hydration was not performed since it does not require such pretreatment prior to use. The same experimental conditions were applied for the artificial membrane experiment. A positive displacement micropipette was used to apply CF and RD formulations either as finite (17.7 or 35.4 μL/ 1.77 cm^2; layered, 17.7 μL and 17.7 μL/ 1.77 cm^2) or infinite dose (1 mL/1.77 cm^2). The applied formulation was spread evenly using the back side of a spatula. Aliquots (500 μL) were withdrawn from the receiver solution at pre-determined time points. Permeation experiments were performed for 8 h.

3.2.4. HPLC Analyses of CF and RD

An aliquot (100 μL) of the RD sample collected at every time point was mixed with an equal volume of internal standard (methylparaben), whereas CF samples were mixed with equal volume of acetonitrile. Samples were then centrifuged at 4°C for 5 min. Each sample was analyzed using an HPLC system (Shimadzu Co., Kyoto, Japan) equipped with column (Inertsil® ODS-3 4.6 mm X 150 mm, GL Sciences Inc., Tokyo, Japan), system controller (SCL-10A), pump (LC-20AD), degasser (DGU-$20A_3$), auto – injector (SIL-20A), column oven (CTO-20A), UV detector (SPD-20A), and analysis

software (LC Solution). The column was maintained at 40°C with the flow rate of the mobile phase at 1.0 mL/min. The mobile phase for RD was acetonitrile and water (25/75, v/v) and 0.1% phosphoric acid and acetonitrile (10/90, v/v) was used for CF. Detection of RD and CF was made at 280 nm and 254 nm, respectively.

3.2.5. Measurement of membrane electrical impedance

Strat-M® (25 mm) discs were mounted in a vertical-type Franz diffusion cell, identically to the conditions described above. Hydration was not performed prior to the measurement of impedance. PBS (pH 7.4) was loaded into the donor and receiver cells. Impedance was first determined for untreated Strat-M® discs using an impedance meter (10 Hz AC, Asahi Techno Lab., Ltd., Yokohama, Japan). The Strat-M® membrane was carefully blotted dry from the donor side, and 10 μL/cm^2 of polyol stock was applied. After 10 mins, polyol was removed from the membrane surface and fresh PBS (pH 7.4) was added, and impedance was determined again.

3.2.6. Statistical analyses

All experimental data were tested for statistical significance ($p < 0.05$) using Student's *t*-test. Pearson's correlation coefficient was used to characterize the relationship between the cumulative amounts of the drug that permeated through the porcine skin and Strat-M®. All data were expressed as mean with a standard error.

3.3. Results

3.3.1. Permeation of drugs under in-use and finite dose conditions

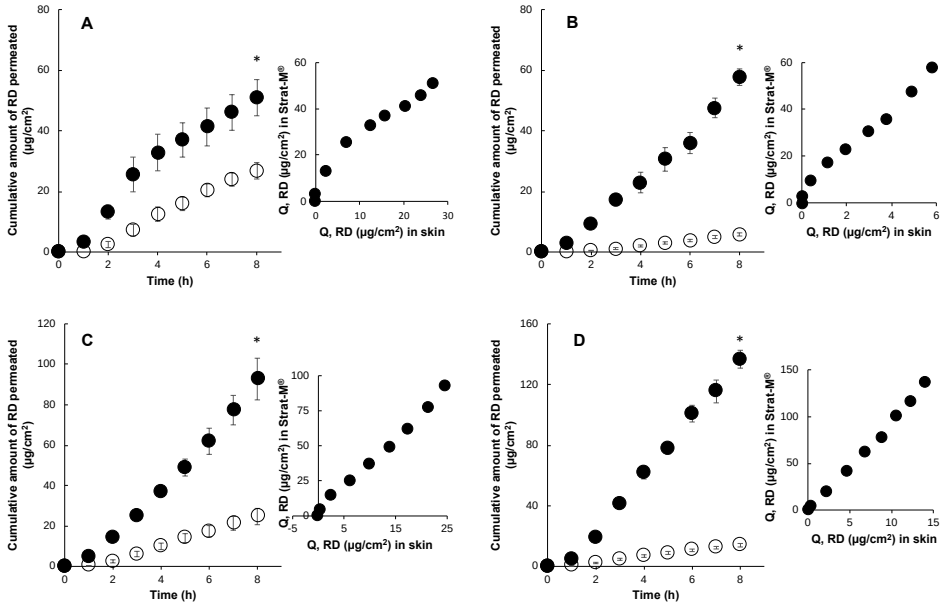

Figure 1. Time course of the cumulative amount of RD permeated through Strat-M® (●) and porcine skin (○) and their correlation under in-use (finite dose) conditions. 1% RD in water single application 10 μL/cm^2 (A), single application 10 μL/cm^2 lotion (B), single application 20 μL/cm^2 lotion (C), layered application 10 -10 μL/cm^2 lotion (D). Each point represents the mean ± S.E. (n=4). Significant difference (*p<0.05) between RD permeation from single application (10 μL/cm^2), layered application, single application (20 μL/cm^2) of lotion, and single application (10 μL/cm^2) aqueous solution through Strat-M® and porcine skin.

Figure 1 illustrates the cumulative amount of RD permeated through porcine skin and Strat-M®. RD formulations were applied in finite doses (single application of 10 μL/cm^2, layered application of 10 μL/cm^2 - 10 μL/cm^2, and single application of 20 μL/cm^2) to simulate in-use conditions, such as layered application and dose in human exposures to the liquids. [20, 23] Permeation of RD from aqueous and complex vehicle-based formulations through porcine skin showed a dose-dependent rank order except for the layered application, where permeation was lowered compared with a single application of 20 μL/cm^2, despite having the same total applied dose.

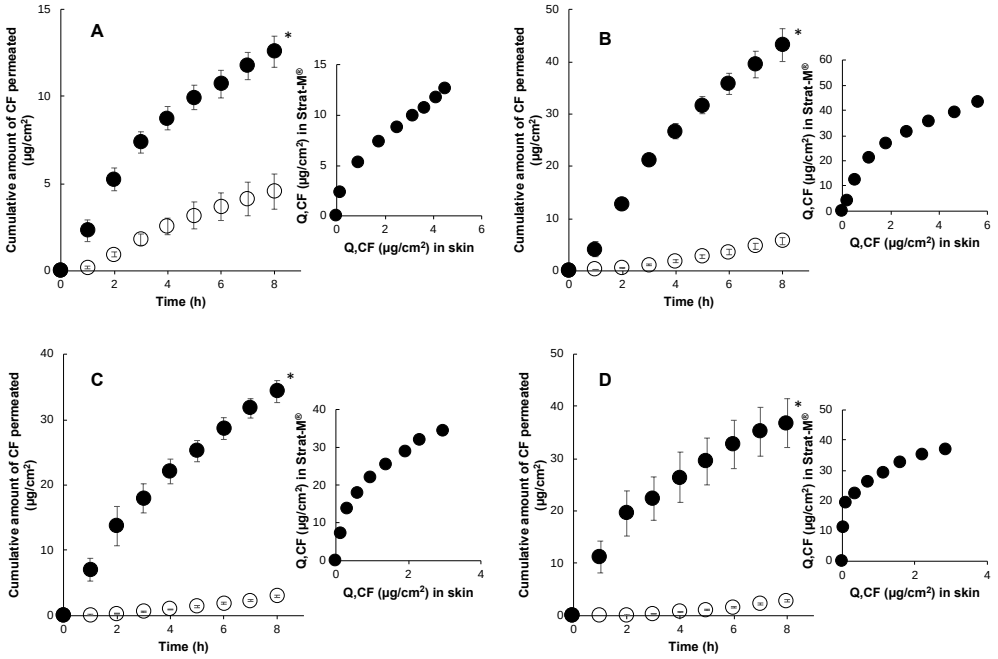

Figure 2. Time course of the cumulative amount of CF permeated through Strat-M® (●) and porcine skin (○) and their correlations under in-use (finite dose) conditions. 1% CF in water (A), 1% CF in 50% polyol (B), 1% CF in 75% polyol (C), and 1% CF in 100% polyol (D). Each point represents the mean ± S.E. (n=4). Significant difference (*p<0.05) between RD permeation through Strat-M® and porcine skin.

Figure 2 illustrates the cumulative amount of CF that permeated through porcine and Strat-M® from a finite dose application of 10 µL/cm^2. Permeation of RD and CF from their aqueous formulations through Strat-M® were in good agreement with permeation data through porcine skin (Figs.1 and 2). Cumulative amount of CF permeated through Strat-M® was significantly higher compared to porcine skin. Permeation ratio (flux, J) was elevated in CF formulations containing higher percentage of polyols. The correlation value of Q through Strat-M® and skin decreased with high polyol concentration (Table 3).

Table 1. Permeation parameters of CF and RD from various formulations through Strat-M® and porcine skin under finite dose conditions

Formulations	*Strat-M®*	*Porcine skin*	Permeation ratio	r^2 ($Q_{Strat\text{-}M®}$ vs. Q_{Skin})
	J (μg/cm²/h)	J (μg/cm²/h)	($J_{Strat\text{-}M®}/J_{Skin}$)	
1% CF in water	0.92 ± 0.05	0.63 ± 0.09	1.45	0.96
1% CF in 50% polyol	5.80 ± 0.27	1.42 ± 0.33	4.07	0.89
1% CF in 75% polyol	2.87 ± 0.27	0.61 ± 0.06	4.73	0.87
1% CF in 100% polyol	3.13 ± 0.53	0.57 ± 0.04	5.47	0.71
1% RD in water	4.08 ± 0.42	2.45 ± 0.12	1.66	0.93

3.3.2. Permeation of drugs under infinite dose conditions

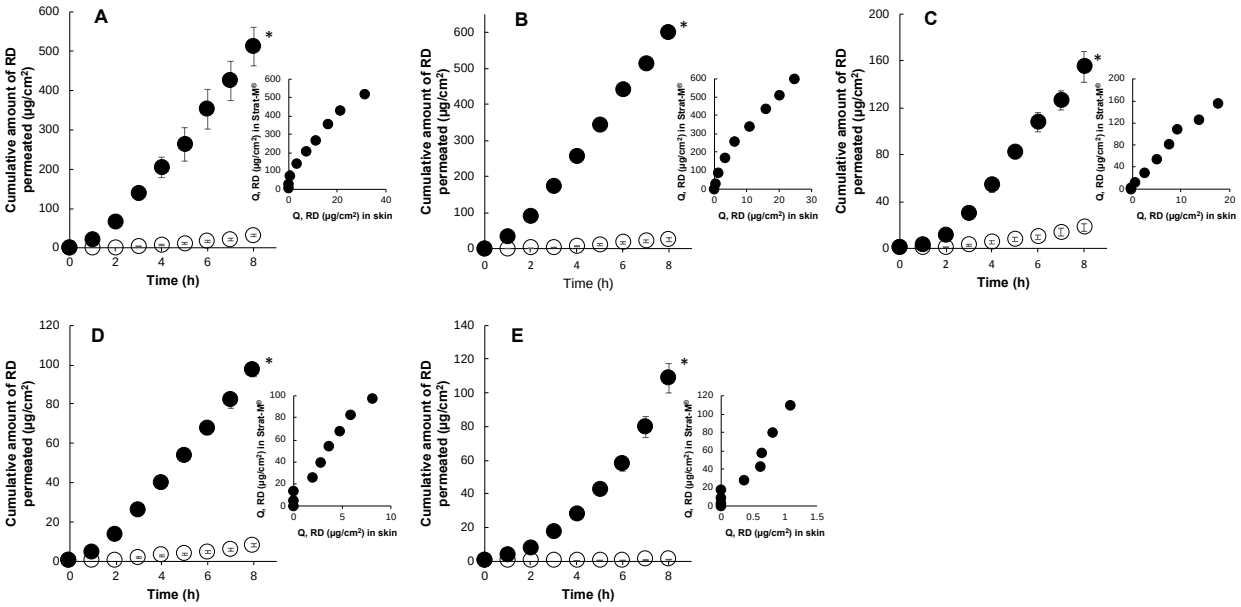

Figure 3. Time course of the cumulative amount of RD permeated through Strat-M® (●) and porcine skin (○) and their correlations under infinite dose conditions. 1% RD in water (A), 2% RD in 19.5% polyol (B), 2% RD in 40% polyol (C), 2% RD in 61.8% polyol (D), and 2% RD in 100% polyol (E). Each point represents the mean ± S.E. (n=4). Significant difference (*p<0.05) between RD permeation through Strat-M® and porcine skin.

Figure 3 presents the permeation of RD through porcine skin and Strat-M® in infinite dose conditions. RD permeation under finite and infinite dose conditions had identical rank orders of permeation [aqueous formulation (0% polyol) > low polyol concentration (19.55) > high polyol concentration (40 and 61.8%) > residual formulation (100% polyol)] through porcine skin, wherein increasing polyol

concentration in formulations corresponds to a decrease in the amount of permeated drug (r^2= 0.98). Overall, the artificial membrane demonstrated a high correlation (r^2=0.94 – 0.98) of permeation with porcine skin for RD.

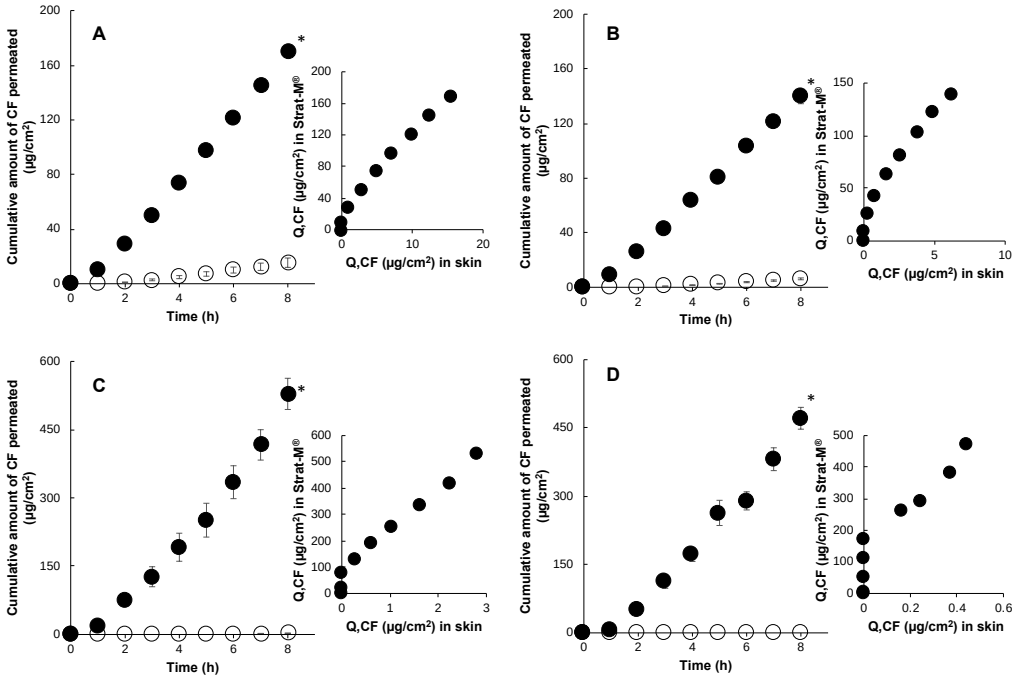

Figure 4. Time course of the cumulative amount of CF permeated through Strat-M® (●) and porcine skin (○) and their correlations under infinite dose conditions. 1% CF in water (A), 1% CF in 50% polyol (B), 1% CF in 75% polyol (C), and 1% CF in 100% polyol (D). Each point represents the mean ± S.E. (n=4). Significant difference (*p<0.05) between RD permeation through Strat-M® and porcine skin.

Figure 4 shows the permeation of CF through porcine skin and Strat-M® in infinite dose conditions. CF permeation through skin in infinite dose conditions had identical rank order [aqueous formulation (0% polyol) > high polyol concentration (50 and 75%) > residual formulation (100% polyol)] of permeation to RD. However, a dissimilar order of permeation for CF was found in permeation experiments through the Strat-M® membrane. The permeation of CF through Strat-M® from formulations with high polyol content and residual formulation was consistently enhanced. The

permeation ratio of CF and RD permeation through Strat-M® and porcine skin is presented in Table 2.

Permeability coefficients were elevated in formulations with high polyol content and residual formulations. No relationship exists between the permeability coefficients of porcine skin and Strat-M®. The permeability coefficient of CF through Strat-M® increased proportionally with the amount of polyol in the formulation, whereas the opposite was observed in experiments using porcine skin with both drugs (Table 2). In RD, a good rank order for aqueous and high polyol formulations (40 and 61.8%) was seen between porcine skin and Strat-M®, however, significant enhancement in permeability was seen in formulation with low polyol content (19.5%).

Table 2. Permeation parameters of CF and RD from various formulations through Strat-M® and porcine skin under infinite dose conditions

Formulations	***Strat-M®***		***Porcine skin***		Permeation ratio ($J_{Strat\text{-}M®}/J_{Skin}$)	r^2 ($Q_{Strat\text{-}M®}$ vs. Q_{Skin})
	P (cm/s) x 10^{-07}	*J* ($\mu g/cm^2/h$)	*P* (cm/s) x 10^{-07}	*J* ($\mu g/cm^2/h$)		
1% CF in water	7.25 ± 0.26	24.01 ± 0.87	1.75 ± 0.36	2.76 ± 0.59	8.71	0.98
1% CF in 50% polyol	4.87 ± 0.17	19.57 ± 0.60	0.36 ± 0.01	1.20 ± 0.15	16.3	0.96
1% CF in 75% polyol	46.6 ± 3.73	93.92 ± 1.66	0.17 ± 0.02	0.59 ± 0.06	160.4	0.97
1% CF in 100% polyol	22.2 ± 1.99	67.97 ± 2.63	0.02 ± 0.005	0.09 ± 0.02	739	0.89
1% RD in water	25.7 ± 1.89	79.99 ± 6.78	6.55 ± 1.75	9.27 ± 0.54	8.63	0.96
2% RD in 19.5% polyol	1327853 ± 44308	95.15 ± 6.92	3.02 ± 1.41	4.60 ± 0.74	20.7	0.97
2% RD in 40% polyol	3.32 ± 0.48	24.39 ± 3.01	0.41 ± 0.07	3.37 ± 0.59	7.24	0.98
2% RD in 61.8% polyol	1.76 ± 0.036	14.53 ± 0.29	0.09 ± 0.03	0.97 ± 0.22	15.03	0.98
2% RD in 100% polyol	3.58 ± 0.22	18.37 ± 2.81	0.01 ± 0.001	0.15 ± 0.01	121	0.94

3.3.3. Effect of polyols on permeation of drugs through Strat-M®

The impact of commonly used polyols as vehicles/solvents in cosmetics on the drug permeation through Strat-M® in in-use conditions was assessed. The electrical impedance of the membrane confirms the integrity of the membrane's barrier property. The compositions of the formulations used in this study represent common solvents used in many cosmetic formulations as well as their formulation dynamics after being

applied onto the skin. The electrical impedance of the membrane was found to be high (≥ 100 kΩ•cm^2), indicating the good barrier properties of its SC-like top layer. Application of polyols, typical solvents in cosmetic formulations, to Strat-M® for 10 min resulted to a significant reduction (92%) in impedance (post-treatment impedance value of 8.02 ± 0.89 kΩ•cm^2) of the membrane.

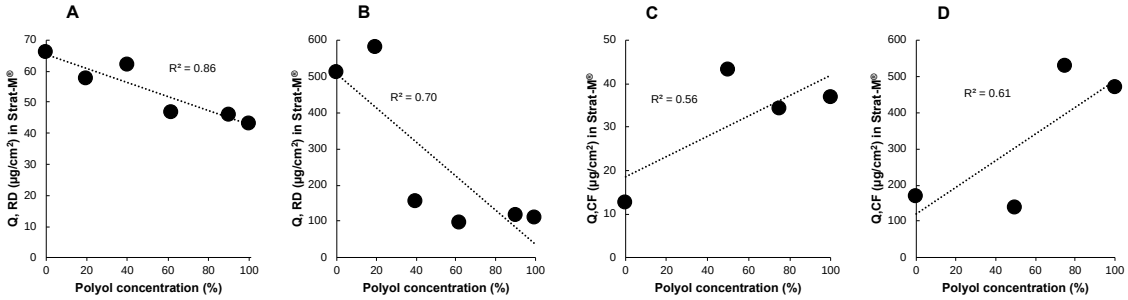

Figure 5. Relationship between cumulative amount permeated at 8 h through Strat-M® and polyol concentration in RD and CF formulations. Finite dose experiment of RD (A), Infinite dose experiment of RD (B), Finite dose experiment of CF (C), and Infinite dose experiment of CF (D).

Figure 5 presents the relationship between the cumulative amount of drug permeated through to Strat-M® and polyol concentration in formulations. Permeation of RD through Strat-M® from a range of polyol concentrations was correlated with polyol concentration in the formulation for finite and infinite dose conditions with an r^2 value of 0.86 and 0.70, respectively. In the case of CF, poor correlations were found in finite (r^2= 0.56) and infinite dose conditions (r^2= 0.61) between the cumulative amount of permeation and polyol concentration (Fig. 5).

3.4. Discussion

This study investigated the usefulness of artificial membrane and its comparative performance with porcine skin in evaluating permeation of cosmetic active compounds under in-use conditions. RD permeation through the artificial membrane predicted accurately the rank order, as expected, for dose conditions, 10 µL/cm^2 and 20

$\mu L/cm^2$, while the permeation of RD from layered application was the highest. Strat-M® was able to discriminate the impact of applied dose and composition of formulation in the permeation of drugs. Excellent correlations (r^2= 0.95 - 1) exist between permeation through the artificial membrane and skin for RD from all applied dose conditions (Fig. 1).

Permeation experiment through Strat-M® using CF, a hydrophilic model drug, was performed to understand its similarities or dissimilarities with porcine skin. Fluxes for CF and RD across Strat-M® were within close range with minimal enhancement ratios of 1.45 and 1.66, respectively (Table 1). However, finite dose experiments of CF with high polyol proportion and simulated residual formulations revealed significantly higher permeation in contrast to its profile in skin. In skin permeation experiments through porcine skin, CF permeation was enhanced by formulation containing 50% polyol while formulations containing 75% and 100% polyol did not enhance permeation and yielded lower skin permeation presumably due to diffusional limitations. CF from its aqueous formulations yielded the lowest permeation whereas formulations containing polyol had significantly higher permeation through Strat-M® . There was a 4 - 5.5-fold increase in flux with a corresponding increase in polyol concentration (Table 1). Polyol in formulation apparently enhanced permeation of CF through Strat-M®.

RD permeation under finite and infinite dose conditions had identical rank order of permeation through porcine skin. High concentrations of polyol on skin reduced the amount of drug permeated. In this case, the residual formulation was deemed sufficiently viscous to cause diffusional limitations. RD permeation through Strat-M® was enhanced in formulation with low polyol concentration (19.5%) while the rest of

the formulations had similar order of permeation (Fig. 3). This suggests similarities in the permeation pathway for RD through porcine ear skin and the artificial membrane.

Despite having identical permeation rank order with RD under infinite dose conditions, a dissimilar order of CF permeation was found through the artificial membrane. Permeation of CF through Strat-M® from formulations of high polyol content and residual formulation was consistently enhanced. This was contradictory to what was observed with skin permeation data, where high proportions of polyol in the residual formulation was found to reduce the thermodynamic activity of CF and RD by solubilization, hence, decreasing the migration of these drugs from polyol to the skin. A lower correlation (r^2= 0.89) was found between CF permeations through skin and the Strat-M® membrane from its residual formulation owing to large variations in their concentration-time point profiles (Fig. 4D).

A relatively high concentration-time point correlation can be observed between Strat-M® and porcine skin in most formulations for both drugs, however, it must be noted that the amount of drug permeating through Strat-M® in all dose conditions is significantly higher than that of skin. Haq et al. have already presented a piece of evidence on the effect of polyol-membrane interaction on drug permeation. The cumulative amount of drug permeated from their control formulation composed of a pure polyol (propylene glycol) through Strat-M® resulted in a 14-fold increase in permeation. [36] Solubilization or lipid extraction of lipophilic structures on its top layer must have caused the similarities in results particularly the increase in permeation despite being applied as finite doses as observed in both studies. Also, the artificial membrane and formulations with high polyol proportions used in this study share lipophilic qualities. Strat-M® lacks the highly organized intercellular structures of the SC, therefore it simply does not mimic the heterogeneous complexity of the SC and

fails to render similar barrier properties to those of the SC to provide the ideal interaction of vehicles with known SC lipids. [81] Since the partitioning of a drug into the skin is dependent on its ability to preferentially 'transfer' from formulation into SC and beyond, this may have been the reason for the unusually high permeation of CF in infinite dose as opposed to how it would permeate from residual formulation through porcine skin. In a porcine skin-based experiment, formulations with high polyol content and residual formulations did not result to an increase in flux. In addition, the diffusivity of chemicals through an artificial membrane is related to its permeation route, with the relatively thin SC-like layer thus providing a low-tortuosity pathway, which is probably the reason for higher permeability of hydrophilic compounds. [40]

Flux for CF from formulation of high polyol content (75%) and residual formulation through Strat-M® was higher by 160 and 739 – fold, respectively. A lower correlation between the permeation enhancement of CF and polyol content through Strat-M® exists. However, it is notable that higher fluxes can be observed in CF formulations containing high polyol content (75% and 100% polyol). Moreover, the permeability coefficient of Strat-M® was enhanced proportionally with increasing concentration of polyol which is probably a concentration-dependent disruption of Strat-M®'s barrier integrity. Hence, an increase in the permeability coefficient can be observed with higher polyol content. Flux of CF through Strat-M® is proportionally enhanced in formulations of high polyol content as well, where applied. Reduction in flux was observed in porcine skin-based experiments where high polyol content could reduce the thermodynamic activity of the permeating drugs. A correct prediction for RD flux was obtained from its aqueous and high polyol content formulations. RD, in its residual formulation, was shown to permeate 121-fold higher through Strat-M®. This finding was also observed when we conducted identical experiment using another

residual formulation containing 90.4% polyol, and it was found that Strat-M® was more permeable (133-fold higher) when formulations with very high polyol content were applied. The application of polyol-based formulations appeared to solubilize the lipid-based SC-like top layer of the artificial membrane. In layered application, the application of the first layer of formulation disrupted the barrier integrity. Hence, it promotes higher RD permeation through Strat-M® from the second layer applied as opposed to the known lowering of permeation through porcine skin in layered application. Water evaporation in layered application typically reduces the thermodynamic activity of RD in the residual formulation. This also supports the unusually high permeation of CF with high polyol and residual formulations in both finite and infinite dose systems through Strat-M®.

The impact of high amounts of polyol remaining on the skin has been established to markedly reduce the permeation of cosmetic active ingredients, and a poor correlation existed between polyol content and permeation. Permeation of RD through Strat-M® from a range of polyol concentrations was inversely correlated with polyol concentrations in the formulation for finite and infinite dose conditions with an r^2 value of 0.86 and 0.70, respectively. RD permeation through Strat-M® is not correlated with polyol concentration in the formulation because the disruption of the barrier integrity is likely to be limited to the lipid-based top-layer of the artificial membrane. The hydrophilic VED-like layer of Strat-M® must have remained intact throughout the experiment and, thus, effectively limited the passage of the lipophilic RD molecule. In the case of CF, despite having lower correlations between the cumulative amount of permeation and polyol concentration in finite (r^2= 0.56) and infinite dose experiments (r^2= 0.61), an enhanced permeation through Strat-M® was observed (Fig. 5). Moreover, high permeation of CF through Strat-M® under finite and

infinite dose conditions was exhibited by formulations with high polyol content. Enhanced permeation of hydrophilic drugs, such as CF, has been previously reported in membranes with reduced electrical impedance due to compromised barrier function. [52, 82]

3.5. Conclusion

High correlations (r^2= 0.94 – 1) in permeation between Strat-M® and porcine skin under finite and infinite dose conditions were observed with RD, whereas these were only observed in finite dose conditions for CF. A poor relationship was obtained between the permeability coefficients of CF and RD through Strat-M®. The amounts of RD and CF that permeated through Strat-M® from complex vehicles was higher in both dose conditions. Similar permeability characteristics between the two membranes can be observed from aqueous formulations.

Permeation of drugs from formulations with high polyol content and residual formulation was increased with an increase in the permeability of the artificial membrane. The barrier integrity of Strat-M® was breached upon contact with high concentrations of polyol by lipid extraction or solubilization of its SC-like top layer, as indicated by the drastic reduction in electrical impedance. The use of Strat-M® in the assessment of dermal permeation of cosmetics may be limited to formulations with low polyol content and finite dose conditions. Assessment of permeation from concurrent application of identical or non-identical formulations (i.e., layered application) and infinite dose conditions with the use of Strat-M® could result in overestimation of the permeation parameters. Good rank order of permeation from formulations with complex vehicle-based formulation applied as finite doses was observed with a

lipophilic compound (RD). Findings from this study suggest the selective potential usefulness of artificial membranes in discriminating the effect of complex vehicle formulations and predicting permeation of cosmetic active ingredients. Further assessments of the permeation of cosmetic active ingredients should be performed by employing other solvent systems and formulations to enhance the applicability of Strat-M® in cosmetic formulation design, optimization and safety assessments.

CONCLUSION

In conclusion, the following were established from the findings of this study.

1. Cosmetics applied by layered application exhibited lower skin permeation of RD compared with a single application despite having the same application dose.
2. Formulations and their components caused varying reductions in RD permeation probably due to changes in the activity coefficient and thermodynamic activity of the active component.
3. Incorporating evaporation kinetics and vehicle-permeant dependent parameters ($K_{sc,ved}$, $D_{sc,ved}$) could improve the precision of mathematical models in predicting permeation and distribution of actives in the skin.
4. The permeability of the artificial membrane to cosmetic actives is enhanced by high polyol content and residual formulation due to disruption in its barrier integrity.
5. Good rank order of permeation from a complex vehicle was observed in the lipophilic compound suggesting selective usefulness of the artificial membrane in discriminating effects of complex vehicles and in predicting permeation of actives.

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REFERENCES

1. Sugibayashi, K., Todo, H., Oda A., 2016. Effect of layered application of different cosmeceutical formulations containing rhododendrol. J. Jpn. Cosmet. Sci. Soc. 40, 259 – 261.
2. Yamaguchi, M., Araki, D., Kanamori, T., Okiyama, Y., Seto, H., Uda, M., Usami, M., Yamamoto, Y., Masunaga, T., Sasa, H., 2017. Actual consumption amount of personal care products reflecting Japanese cosmetic habits. J. Toxicol. Sci. 42, 797 – 814.
3. Sasaki, M., Kondo, M., Sato, K., Umeda, M., Kawabata, K., Takahashi, Y., Suzuki, T., Matsunaga, K., and Inoue, S., 2014. Rhododendrol, a depigmentation-inducing phenolic compound, exerts melanocyte cytotoxicity via a tyrosinase-dependent mechanism. Pigm. Cell Melanoma R. 27, 754–763.
4. Abe, Y., Okamura, K., Kawaguchi, M., Hozumi, Y., Aoki, H., Kunisada, T., Ito, S., Wakamatsu, K., Matsunaga, K., Suzuki, T., 2016. Rhododendrol-induced leukoderma in a mouse model mimicking Japanese skin. J. Dermatol. Sci. 81, 35-43.
5. Gabe, Y., Miyaji, A., Kohno, M., Hachiya, A., Moriwaki, S., Baba, T., 2018. Substantial evidence for the rhododendrol-induced generation of hydroxyl radicals that causes melanocyte cytotoxicity and induces chemical leukoderma. J. Dermatol. Sci. 91, 311-316.
6. Tokura, Y., Fujiyama, T., Ikeya, S., Tatsuno, K., Aoshima, M., Kasuya, A., Ito, T., 2015. Biochemical, cytological, and immunological mechanisms of rhododendrol-induced leukoderma. J. Dermatol. Sci. 77, 146 – 149.
7. Mesko, M.F., Novo, D.L.R., Costa, V.C., Henn, A.S., Flores, E.M.M., 2020. Toxic and potentially toxic elements determination in cosmetics used for make-up: a critical review. Anal. Chim. Acta 1098, 1 – 26.
8. Alves, V.M., Muratov, E.N., Zakharov, A., Muratov, N.N., Andrade, C.H., Tropsha, A., 2018. Chemical toxicity prediction for major classes of industrial chemicals: is it possible to develop universal models covering cosmetics, drugs, and pesticides? Food Chem Toxicol. 112, 526 – 534.
9. Almeida, A., Sarmento, B., Rodrigues, F., 2017. Insights on in vitro models for safety and toxicity assessment of cosmetic ingredients. Int. J. Pharm. 518, 178 – 185.

10. Desprez, B., Dent, M., Keller, D., Klaric, M., Ouédraogo, G., Cubberley, R., Duplan, H., Eilstein, J., Ellison, C., Grégoire, S., Hewitt, N.J., Jacques-Jamin, C., Lange, D., Roe, A., Rothe, H., Blaauboer, B.J., Schepky, A., Mahony, C., 2018. A strategy for systemic toxicity assessment based on non-animal approaches: the cosmetics Europe long range science strategy programme. Toxicol In Vitro 50, 137 – 146.
11. Plošnik, A., Zupan, J., Vračko, M., 2015. Evaluation of toxic endpoints for a set of cosmetic ingredients with CAESAR models. Chemosphere 120, 492 – 499.
12. Goebel, C., Aeby, P., Ade, N., Alépée, N., Aptula, A., Araki, D., Dufour, E., Gilmour, N., Hibatallah, J., Keller, D., Kern, P., Kirst, A., Marrec-Fairley, M., Maxwell, G., Rowland, J., Safford, B., Schellauf, F., Schepky, A., Seaman, C., Teichert, T., Tessier, N., Teissier, S., Weltzein, H.U., Winkler, P., Scheel, J., 2012. Guiding principles for the implementation of non-animal safety assessment approaches for cosmetics: skin sensitization. Regul. Toxicol. Pharmacol. 63, 40 – 52.
13. Ellison, C.A., Blackburn, K.L., Carmichael, P.L., Clewel, H.J., Cronin, M.T.D., Desprez, B., Escher, S.E., Ferguson, S.S., Grégoire, S., Hewitt, N.J., Hollnagel, H.M., Klaric, M., Patel, A., Salhi, S., Schepky, A., Schmitt, B.G., Wambaugh, J.F., Worth, A., 2019. Challenges in working towards an internal threshold of toxicological concern (iTTC) for use in the safety assessment of cosmetics: discussions from the cosmetics Europe iTTC working group workshop. Regul. Toxicol. Pharmacol. 103, 63 – 72.
14. Williams, F.M., Rothe, H., Barrett, G., Chiodini, A., Whyte, J., Cronin, M.T.D., Monteiro-Riviere, N.A., Plautz, J., Roper, C., Westerhout, J., Yang, C., Guy, R.H., 2016. Assessing the safety of cosmetic chemicals: consideration of a flux decision tree to predict dermally delivered systemic dose for comparison with oral TTC (threshold of toxicological concern). Regul. Toxicol. Pharmacol. 76, 174 – 186.
15. Ates, G., Steinmetz, F.P., Doktorova, T.Y., Madden, J.C., Rogiers, V., 2016. Linking existing in vitro dermal absorption data to physicochemical properties: contribution to the design of a weight-of-evidence approach for the safety evaluation of cosmetic ingredients with low dermal bioavailability. Regul. Toxicol. Pharmacol. 76, 74 – 78.

16. Oshizaka, T., Kikuchi, K., Radhum, W.R., Todo, H., Hatanaka, T., Wierzba, K., Sugibayashi, K., 2014. Estimation of skin concentrations of topically applied lidocaine at each depth profile. Int. J. Pharm. 475, 292 – 297.
17. Sugibayashi, K. 2017. Chemical disposition in skin. In: Sugibayashi, K., (ed.) Skin permeation and disposition of therapeutic and cosmeceutical compounds. Springer Japan. pp 55-68.
18. Sugibayashi, K., Todo, H., Oshizaka, T., Owada, Y., 2010. Mathematical model to predict skin concentration of drugs: toward utilization of silicone membrane to predict skin concentration of drugs as an animal testing alternative. Pharm Res. 27, 134-142.
19. Selzer, D., Abdel-Mottaleb, M.M., Hahn, T., Schaefer, U., Neumann, D., 2013. Finite and infinite dosing: difficulties in measurements, evaluations and predictions. Adv. Drug Deliv. Rev. 65, 278-294.
20. SCCS/1602/18, 2018. The SCCS's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation, 10th revision. Scientific Committee on Consumer Safety. pp 11-16.
21. SCCS/1416/11, 2012. The SCCS's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation, 7th revision. Scientific Committee on Consumer Safety. pp 68.
22. SCCS/1358/10, 2010. Basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients. Scientific Committee on Consumer Safety. pp 8.
23. Arce, F.V., Asano, N., Yamashita, K., Oda, A., Uchida, T., Sano, T., Todo, H., Sugibayashi, K., 2019. Effect of layered application on the skin permeation of a cosmetic active, rhododendrol. J. Toxicol. Sci. 44, 1-11.
24. Otto, A., Wiechers, J.W., Kelly, C.L., Hadgraft, J., du Plessis, J., 2008. Effect of penetration modifiers on the dermal and transdermal delivery of drugs and cosmetic active ingredients. Skin Pharmacol. Physiol. 21, 326-334.
25. Poulsen, B.J., 1973. Design of topical drug products: biopharmaceutics. In: Ariëns, E.J. (ed.). Drug Design. Academic press, pp. 149-192.
26. Benford, D.J., Cocker, J., Sartorelli, P., Schneide, T., van Hemmen, J., Firth, J.G., 1999. Dermal route in systemic exposure. Scand. J. Work Environ. Health. 25, 511–520.

27. European Commission, 2004. Guidance document on dermal absorption. Sanco/222/2000 rev. 7.pp 1–15.
28. Rothe, H., Obringer, C., Manwaring, J., Avci, C., Wargniez, W., Eilstein, J., Hewitt, N., Cubberley, R., Duplan, H., Lange, D., Jacques-Jasmin, C., Klaric, M., Schepky, A., Grégoire, S., 2017. Comparison of protocols measuring diffusion and partition coefficients in the stratum corneum. J. Appl. Toxicol. 37, 806 – 816.
29. Mitragotri, S., Anissimov, Y.G., Bunge, A.L., Frasch, H.F., Guy, R.H., Hadgraft, J., Kasting, G.B., Lane, M.E., Roberts, M.S., 2011. Mathematical models of skin permeability: an overview. Int. J. Pharm. 418, 115 – 129.
30. Lehman, P.A., 2014. A simplified approach for estimating skin permeation parameters from in vitro finite dose absorption studies. J. Pharm. Sci. 103, 4048 – 4057.
31. Sloan, K.B., Koch, S.A.M., Siver, K.G., Flowers, F.P., 1986. Use of solubility parameters of drug and vehicle to predict flux through skin. J. Investigativ. Dermatol. 87, 244 – 252.
32. Hansen, S., Lehr, C.M., Schaefer, U.F., 2013. Improved input parameters for diffusion models of skin absorption. Adv. Drug. Deliv. Rev. 65, 251 – 264.
33. Kasting, G.B., Miller, M.A., LaCount, T.D., Jaworska, J., 2019. A composite model for the transport of hydrophilic and lipophilic compounds across the skin: steady-state behavior. J. Pharm. Sci. 108, 337 – 349.
34. Haq, A., Dorrani, M., Goodyear, B., Joshi, V., Michniak-Kohn, B., 2018. Membrane properties for permeability testing: skin versus synthetic membranes. Int. J. Pharm. 539, 58 – 64.
35. Simon, A., Amaro, M.I., Healy, A.M., Cabral, L.M., de Sousa, V.P., 2016. Comparative evaluation of rivastigmine permeation from a transdermal system in the Franz cell using synthetic membranes and pig ear skin with in vivo-in vitro correlation. Int. J. Pharm. 512, 234 – 241.
36. Haq, A., Goodyear, B., Ameen, D., Joshi, V., Michniak-Kohn, B., 2018. Strat-M® synthetic membrane: permeability comparison to human cadaver skin. Int. J. Pharm. 547, 432-437.
37. Merck, Pioneering skin testing. Experience the unmatched predictability of Strat-M membrane, Lit. no. MK-BR2545EN ver. 1.0. Merck KGaA, Darmstadt, Germany. 2018.

38. Sugibayashi, K., Yusuf, E., Todo, H., Dahlizar, S., Sakdiset, P., Arce, F.J., See, G.L., 2019. Halal cosmetics: a review on ingredients, production, and testing methods. Cosmetics. 6, 1-17.
39. Hatanaka, T., Inuma, M., Sugibayashi, K., Morimoto, Y., 1990. Prediction of skin permeability of drugs. I. Comparison with artificial membrane. Chem. Pharm. Bull. 38, 3452-3459.
40. Uchida, T., Kadhum, W., Kanai, S., Todo, H., Oshizaka, T., Sugibayashi, K., 2015. Prediction of skin permeation by chemical compounds using the artificial membrane, Strat-M™. Eur. J. Pharm. Sci. 67, 113-118.
41. Kwan, Y.H., Tung, Y.K., Kochlar, J.S., Li, H., Poh, A., Kang, L., 2014. Skin permeation of cosmetics. In: Kwan, Y.H., Tung, Y.K., Kochlar, J.S., Li, H., Poh, A., Kang, L. (Eds.), Handbook of cosmeceutical excipients and their safeties. Woodhead Publishing. pp. 31-34.
42. Simon, G., Maibach, H. I., 2000. The pig as an experimental animal model of percutaneous permeation in man: qualitative and quantitative observations--an overview. Pharmacol. Appl. Skin Physiol. 13, 229-34.
43. Cilurzo, F., Minghetti, P., Sinico, C., 2007. Newborn pig skin as model membrane in in vitro drug permeation studies: a technical note. AAPS PharmSciTech. 8, 97-100.
44. Aoyama, Y., Ito, A., Suzuki, T., Tanemura, A., Nishigori, C., Ito, M., Katayama, I., Sugiura, S., Matsunaga, K., 2014. The first epidemiological report of rhododendrol-induced leukoderma in Japan based on a nationwide survey. Jpn. J. Dermatol. 124, 2095-2109.
45. Arase, N., Yang, L., Tanemura, A., Yang, F., Suenaga, T., Arase, H., Katayama, I., 2016. The effect of rhododendrol inhibition of NF-kB on melanocytes in the presence of tyrosinase J. Dermatol. Sci. 83, 148-64.
46. Okura, M., Yamashita, T., Ishii-Osai, Y., Yoshikawa, M., Sumikawa, Y., Wakamatsu, K., Ito, S., 2015. Effects of rhododendrol and its metabolic products on melanocytic cell growth. J. Dermatol. Sci. 80, 142-149.
47. Karadzovska, D., Brooks, J.D., Monteiro-Riviere, N.A., Riviere, J.E., 2013. Predicting skin permeability from complex vehicles. Adv. Drug Deliv. Rev. 65, 265 – 277.
48. Hojerova, J., Perackova, Z., Berankova, M., 2017. Margin of safety for two UV filters estimated by in vitro permeation studies mimicking consumer habits:

Effects of skin shaving and sunscreen reapplication. Food Chem. Toxicol. 103, 66 – 78.

49. Zhang, X., Yu, Y., Gu, Y., Li, X., Zhang, X., Yu, Y., 2017. *In vitro* determination of transdermal permeation of synthetic musks and estimated dermal uptake through usage of personal care products. Chemosphere. 173, 417 – 424.
50. OECD, 2004a. Organization for Economic Cooperation and Development. Guideline 428: Skin Absorption. OECD Press. P 8.
51. Flaten, G., Palac, Z., Engesland, A., Filipovic-Grcic, J., Vanic, Z., 2015. *In vitro* skin models as a tool in optimization of drug formulation. Eur. J. Pharm. Sci. 75, 10-24.
52. Todo, H., 2017. Transdermal Permeation of Drugs in Various Animal Species. Pharmaceutics. 3, 9-33.
53. Barbero, AM and Frasch, HF., 2009. Pig and guinea pig skin as surrogates for human in vitro penetration studies: a quantitative review. Toxicol. In Vitro. 23, 1-13.
54. OECD, 2004b. Organization for Economic Cooperation and Development. Guidance document for the conduct of skin absorption studies number 28. OECD Press. pp 14.
55. OECD, 2011. Organization for Economic Cooperation and Development. Guidance Notes on Dermal Absorption. Series on Testing and Assessment number 156. OECD Press. pp 26.
56. Akomea, F., Nazir, T., Martin, GP., Brown, MB., 2004. Effect of heat on the percutaneous absorption and skin retention of three model penetrants. Eur. J. Pharm. Sci. 21, 337-345.
57. Hao, J., Ghosh, P., Newman, B., Kasting, G., Raney, S., 2016. Heat effects on drug delivery across human skin. Expert Opin. Drug Deliv. 13, 755- 768.
58. Coldman, M., Poulsen, B., Higuchi, T., 1969. Enhancement of percutaneous absorption by the use of volatile: nonsolvent system as vehicles. J. Pharm. Sci. 58, 1098-1102.
59. Santos, P., Watkinson, A., Hadgraft, J., Lane, M., 2010. Oxybutynin permeation in skin: The influence of drug and solvent activity. Int. J. Pharm., 384, 67-72.
60. Lane, M., Hadgraft, J., Oliveira, G., Viiera, R., Mohammed, D., Hirata, K., 2012. Rational formulation Design. Int. J. Cosmet. Sci. 34, 496- 501.

61. Oliveira, G., Hadgraft, J., Lane, M.E., 2012. The influence of volatile solvents on transport across model membranes and human skin. Int. J. Pharm. 435, 38-49.
62. Tanaka, S., Takashima, Y., Murayama, H., Tsuchiya, S., 1985. Studies on drug release from ointments. V. Release of hydrocortisone butyrate propionate from topical dosage forms to silicone rubber. Int. J. Pharm. 27, 29- 38.
63. Wang, T., Kasichayanula, S., Gu, X., 2006. *In vitro* permeation of repellent DEET and sunscreen oxybenzone across three artificial membranes. Int. J. Pharm. 310, 110- 117.
64. Gu, X., Wang, T., Collins, D., Kasichayanula, S. and Burczynski, F., 2005. *In vitro* evaluation of concurrent use of commercially available insect repellent and sunscreen preparation. Br. J. Dermatol. 152, 1263- 1267.
65. Karakatsani, M., Dedhiya, M., Plakogiannis, FM., 2010. The effect of permeation enhancers on the viscosity and the release profile of transdermal hydroxyropyl methylcellulose gel formulations containing diltiazem HCl. Drug Dev. Ind. Pharm. 36, 1195-206.
66. Ross, J., Shah, J., 2000. Reduction in skin permeation of N, N-diethyl-m-toluamide (DEET) by altering the skin/vehicle partition coefficient. J. Control. Release 67, 211-21.
67. Hadgraft, J., Lane, M., 2016. Advanced topical formulations. Int. J. Pharm., 514, 52-57.
68. Gajjar, R., Miller, M., Kasting, G., 2013. Evaporation of volatile organic compounds from human skin *in vitro*. Ann. Occup. Hyg. 57, 853 – 865.
69. Magnusson, B.M., Pugh, W.J., Roberts, M.S., 2004. Simple rules defining the potential of compounds for transdermal delivery or toxicity. Pharm. Res. 21, 1047 – 1054.
70. Wiechers J.W., Watkinson, A.C., Cross, S.E., Roberts, M.S., 2012. Predicting skin penetration of actives from complex cosmetic formulations: an evaluation of inter formulation and inter active effects during formulation optimization for transdermal delivery. Int. J. Cosmet. Sci. 34, 525 – 535.
71. Potts, R., Guy, R., 1992. Predicting skin permeability. Pharm. Res. 9, 663-669.
72. Dias, M., Hadgraft, J., Lane, M.E., 2007. Influence of membrane-solvent-solute interactions on solute permeation in skin. Int. J. Pharm. 340, 65 – 70.

73. Oshizaka, T., Kikuchi, K., Kadhum, W., Todo, H., Hatanaka, T., Wierzba, K., Sugibayashi, K., 2014. Estimation of skin concentrations of topically applied lidocaine at each depth profile. Int J. Pharm. 475, 292-297.
74. Hada, N., Hasegawa, T., Takahashi, H., Ishibashi, T., Sugibayashi, K., 2005. Culture skin loaded with tetracycline HCl and chloramphenicol as dermal delivery system: mathematical evaluation of the cultured skin containing antibiotics. J Control. Release. 108, 341-350.
75. Ishii, H., Todo, H., Sugibayashi, K., 2010. Effect of thermodynamic activity on skin permeation and skin concentration of triamcinolone acetonide. Chem. Pharm. Bull. 58, 556 – 561.
76. Sato, K., Mitsui, N., Hasegawa, T., Sugibayashi, K., Morimoto, Y., 2001. Potential usefulness of solubility index for prediction of the skin permeation rate of 5-ISMN from pressure-sensitive adhesive tape. J Control. Release. 73, 269-277.
77. Wiechers, J.W., Kelly, C.L., Blease, T.G., Dederen, J.C., 2004. Formulating for efficacy. Int. J. Cosmet. Sci. 26, 173-182.
78. Oliveira, G., Hadgraft, J., Lane, M.E., 2012. The influence of volatile solvents on transport across model membranes and human skin. Int. J. Pharm. 435, 38 – 49.
79. Schaefer, U., Lippold, B., Leopold, C., 2007. Formulation issues, in: Roberts, M, Walters, K. (Eds.). Dermal absorption and toxicity assessment. Informa healthcare. pp 117-120.
80. Uchida, T., Nishioka, K., Anzu, M., Yakumaru, M., Sano, T., Todo, H., Sugibayashi, K., 2016. Effect of esters on the permeation of chemicals with different polarities through synthetic artificial membranes using high-throughput diffusion cell array. Chem. Pharm. Bull. 64, 1597-1606.
81. Karadzovska, D., Riviere, J., 2013. Assessing vehicle effects on skin absorption using artificial membrane assays. Eur. J. Pharm. Sci. 50, 569-576.
82. Sakdiset, P., Kitao, Y., Todo, H., Sugibayashi, K., 2017. High-throughput screening of potential skin penetration-enhancers using stratum corneum lipid liposomes; preliminary evaluation for different concentrations of ethanol. J. Pharm. 1-11.