International Journal of Pharmaceutics 637 (2023) 122891

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Effects of storage conditions on permeability and electrical impedance properties of the skin barrier

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A B S T R A C T

The aim of this study was to investigate the effect of various skin preservation protocols on *in vitro* drug permeation, epidermal-dermal drug distribution, and electrical impedance properties of skin membranes. Acyclovir (AC) and methyl salicylate (MS) were selected as model drugs due to their different physicochemical properties and skin metabolic profiles. In particular, AC is relatively hydrophilic (logP −1.8) and not expected to be affected by skin metabolism, while MS is relatively lipophilic (logP 2.5) and susceptible to metabolism, being a substrate for esterase residing in skin. Skin from pig ears was used and freshly excised into split-thickness membranes, which were divided and immediately stored at five different storage conditions: a) 4 °C overnight (fresh control), b) 4 °C for 4 days, c) and d) −20 °C for 6 weeks and one year, respectively, and e) −80 °C for 6 weeks. Based on the combined results, general trends are observed showing that fresh skin is associated with lower permeation of both model drugs and higher skin membrane electrical resistance, as compared to the other storage conditions. Interestingly, in the case of fresh skin, significantly lower amounts of MS are detected in the epidermis and dermis compartments, implying higher levels of ester hydrolysis of MS (i.e., higher esterase activity). In line with this, the concentration of salicylic acid (SA) extracted from the dermis is significantly higher for fresh skin, as compared to the other storage conditions. Nevertheless, for all storage conditions, substantial amounts of SA are detected in the receptor medium, as well as in the epidermis and dermis, implying that esterase activity is maintained to some extent in all cases. For AC, which is not expected to be affected by skin metabolism, freeze storage (protocols c-e) is observed to result in higher accumulation of AC in the epidermis, as compared to the case of fresh skin, while the AC concentration in dermis is unaffected. These observations can be rationalized primarily by the observed lower permeability of fresh skin towards this hydrophilic substance. Finally, a strong correlation between AC permeation and electrical skin resistance is shown for individual skin membranes irrespective of storage condition, while the corresponding correlation for MS is inferior. On the other hand, a strong correlation is shown for individual membranes between MS permeation and electrical skin capacitance, while a similar correlation for AC is lower. The observed correlations between drug permeability and electrical impedance open up for standardizing *in vitro* data for improved analysis and comparisons between permeability results obtained with skin stored at different conditions.

1. Introduction

The protocols currently used for experimental *in vitro* studies on percutaneous penetration of drugs varies to a large extent due to diverse study objectives and different methodologies used, which in certain cases obscures the interpretation of permeability data. One main problem is that it is practically challenging to perform *in vitro* experiments on fresh skin obtained during surgery or directly after skin harvesting from animal sources. Normally, this issue is solved by storing skin tissue until the experiments can be performed. However, the guidelines for storage conditions of skin tissue are not always clear and several different recommendations exist in the literature (Barbero and Frasch, 2016; Nielsen et al., 2011). Several studies have evaluated the effect of skin storage on *in vitro* skin penetration of various molecules, but the literature is full of unclear and contradictory results; e.g., see Barbero and Frasch for an overview of several studies (Barbero and Frasch, 2016). One underlying factor contributing with ambiguity is the inherently great biological variability associated with *in vitro* percutaneous penetration, which makes it very complex to conclusively establish if different storage conditions have a significant effect or not. To illustrate the complexity of this issue, a summary of a few previous investigations follows.

Swarbrick et al. investigated the penetration of proxicromil (MW 302 g/mol, logP 4.8) across heat separated human skin from three donors in prolonged diffusion experiment (over 80 h) and compared the influence of freeze storage at −17 °C (Swarbrick et al., 1982). They found that freeze stored skin had significantly elevated permeability at 40 h and 60 h in the case of two donors, as compared to fresh skin from the same donors, while no statistically difference was observed for skin

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https://doi.org/10.1016/j.ijpharm.2023.122891
Received 8 February 2023; Received in revised form 22 March 2023; Accepted 23 March 2023
Available online 29 March 2023

collected from a third donor (Swarbrick et al., 1982). Further, they reported that drying freshly prepared skin overnight at 25 % relative humidity and ambient temperature, followed by storage in aluminum foil at 1 °C, resulted in similar permeability characteristics as compared to fresh skin (Swarbrick et al., 1982). In another study, the penetration of T-2 toxin (MW 467 g/mol, logP 2.0) across full-thickness human and monkey skin membranes was shown to increase significantly after storage at −60 °C (up to 10 days), as compared to storage at 4 °C (also up to 10 days) (Kemppainen et al., 1986). Dennerlein et al. investigated transdermal transport of anisole (MW 108 g/mol, logP 2.2), cyclohexanone (MW 98 g/mol, logP 0.82) and 1,4-dioxane (MW 88 g/mol, logP −0.26) across freshly prepared split-thickness human skin membranes and compared with membranes stored for 4 and 30 days at −20 °C and concluded that there was no significant effect of the storage conditions on the fluxes of these molecules (Dennerlein et al., 2013). Nielsen et al. employed full-thickness human skin to investigate the influence of freeze storage (-20 °C or −80 °C for three weeks) on the permeability of caffeine (MW 194 g/mol, logP −0.63), while the *stratum corneum* (SC) structure was studied with multiphoton excitation fluorescence microscopy measurements (Nielsen et al., 2011). They found that caffeine permeability across fresh skin and skin stored at −20 °C was identical in spite of clear structural alterations of the SC barrier (Nielsen et al., 2011). However, the caffeine penetration was significantly elevated for skin stored at −80 °C and this finding was correlated with even more severe alterations of the SC structure (Nielsen et al., 2011). Sintov and Greenberg also employed caffeine and investigated the effect of freeze storage (-20 °C for 1 week) on its permeability across full-thickness skin membranes, but utilized pig, rabbit, and rat skin instead of human skin (Sintov and Greenberg, 2014). Somewhat surprisingly, they found that caffeine penetration was consistently lower at statistically significant levels for frozen rat skin compared to fresh skin, while the opposite relationship was observed for pig skin (Sintov and Greenberg, 2014). The caffeine permeability across rabbit skin, on the other hand, was not affected by freeze storage to any significant degree (Sintov and Greenberg, 2014). Ahlstrom et al. performed a study on the penetration of hydrocortisone (MW 362 g/mol, logP 1.8) through full-thickness canine skin and demonstrated successively increased permeability and decreased lag-time following freeze storage at −20 °C for 1, 4, 8, and 12 months (Ahlstrom et al., 2007). Barbero and Frasch used heat-separated human skin and investigated the effect of freeze storage at −85 °C for up to 18 months (with and without 10% glycerol as cryoprotective agent) on the permeability of diethyl phthalate (MW 222 g/mol, logP 2.7) and observed identical results for all conditions in terms of steady state flux and lag time (Barbero and Frasch, 2016). Further, their study also compared the penetration of caffeine through frozen skin (stored for 4, 8, and 12 months) and found no significant changes of the caffeine transport (Barbero and Frasch, 2016). However, their study lacks experimental data on penetration of caffeine through fresh skin, which precludes any comparison between fresh and frozen skin (Barbero and Frasch, 2016). Jacques-Jamin et al. studied the permeation and skin distribution of *trans*-cinnamic acid (MW 148 g/mol, logP 2.1), benzoic acid (MW 122 g/mol, logP 1.9), and 6-methylcoumarin (160 g/mol, log P 2.3) using split-thickness membranes from four human donors and compared the results obtained with either fresh membranes or membranes stored at −20 °C for 8–12 weeks (Jacques-Jamin et al., 2017). They concluded that the skin permeation and bioaccumulation of these chemicals was essentially unaffected by the freeze storage protocol, while some statistical differences were observed indicating that frozen skin was associated with lower transdermal transport and bioaccumulation in SC, epidermis, and dermis (Jacques-Jamin et al., 2017). Meira et al. investigated the effect of freeze storage at −20 °C for 30, 60, and 90 days on the skin absorption and permeation of penciclovir (MW 523 g/mol, logP −1.4) from a commercial formulation (Meira et al., 2020). They employed full-thickness skin membranes from pig ears and concluded that both epidermal and dermal drug concentrations, as well as the permeation rate, were similar for fresh membranes and membranes stored for 30 days at −20 °C (Meira et al., 2020). However, freeze storage for 60 and 90 days resulted in significantly elevated values of these parameters, as compared to fresh skin (Meira et al., 2020). Mojsiewicz-Pienkowska et al. studied the percutaneous permeation and bioaccumulation of the cyclic siloxane octamethylcyclotetrasiloxane (MW 296 g/mol, logP 7.0) using full-thickness human skin membranes, which were employed as nonfrozen or freeze stored at −20 °C for 2 weeks (Mojsiewicz-Pieńkowska et al., 2022). They concluded that no significant differences were observed, neither regarding the siloxane bioaccumulation in SC, epidermis, and dermis, nor the amount permeated across the skin membranes (Mojsiewicz-Pieńkowska et al., 2022).

Taken together, it is clear that the influence of skin storage condition on the *in vitro* skin permeability and the drug distribution inside the skin membrane represent issues with great complexity where several parameters may influence the outcome of any study. Despite several attempts to establish the effect of different storage conditions on the skin barrier permeability, it can be concluded that the results are contradictory. Further, to the best of our knowledge, the effect of different storage conditions on the skin membrane electrical properties has not been investigated by electrical impedance spectroscopy (EIS) before. This is a useful technique, which is often employed to investigate the effect of barrier damage (White et al., 2013) or how various external parameters, such as hydration (Björklund et al., 2013b; Morin et al., 2020) or pH (Jankovskaja et al., 2021), influence the electrical properties of the skin barrier. With this as background, the aim of this study is to investigate the influence of five relevant skin storage conditions on the permeability and electrical impedance properties of split-thickness pig skin membranes. The storage conditions included: (a) 4 °C overnight (used as control and referred to as fresh skin), (b) 4 °C for 4 days, (c) −20 °C for 6 weeks, (d) −20 °C for one year, (e) −80 °C for 6 weeks. In particular, the study aims at investigating if EIS can be utilized as an *in vitro* tool for correlating changes of the permeability characteristics with changes of the electrical properties of skin membranes after storage at different conditions, which could be useful for reducing interlaboratory variability. To enable a more general analysis, we employ two model drugs with different physicochemical properties and metabolic profiles. Acyclovir (AC) is a hydrophilic substance (MW 225 g/mol, logP −1.8) not expected to be influenced by skin metabolism (Volpato et al., 1998). Methyl salicylate (MS), on the other hand, is a lipophilic molecule (MW 152, logP 2.5) and a known substrate for esterase residing in skin, which converts MS into salicylic acid (SA) via ester hydrolysis (Cross et al., 1998; Guzek et al., 1989; Megwa et al., 1995; Potts et al., 1989). The effect of storage condition on the skin metabolism of MS is, however, less investigated. Taking this into account, we investigate if the skin metabolism of MS is subject to changes due to the different storage conditions by analyzing the MS and SA amounts found in the receptor solution over time, as well as their partitioning into the epidermis and the dermis compartments after the permeation experiments.

2. Materials and methods

2.1. Materials

Acyclovir (AC) was obtained from Jucker Pharma (Täby, Sweden). Methyl salicylate (MS), salicylic acid (SA), AC pharmaceutical secondary standard, citric acid, and sodium citrate was purchased from Sigma Aldrich (Solna, Sweden). Sodium chloride, sodium hydrogen phosphate, monopotassium phosphate, acetonitrile, methanol, and acetic acid were purchased from VWR International (Spånga, Sweden). Porcine ears were obtained from Strömbecks Gårdsslakt och Chark (Illstorp, Sweden) and two-week skin culture medium was purchased from Biopredic International (Saint Grégoire, France).

2.2. Skin preparation and storage

Porcine ears were obtained fresh from a local abattoir (Strömbecks Gårdsslakt och Chark, Illstorp, Sweden). Before use, the skin was rinsed with cold running tap water and the inner ear skin was dermatomed (Padgett dermatome model B, Integra Lifesciences, Cincinnati, USA) to a thickness of approximately 500 μm. Membranes were divided into five groups, which were treated according to one of the five options: (a) skin membranes, slightly moistened by demineralized water, placed between two aluminum foil sheets and stored in a refrigerator (4 °C) overnight (referred to as Fresh); (b) skin membranes placed with dermis side down on a filter paper moistened with two-week skin culture medium and kept in refrigerator (4 °C) for 4 days (referred to as Fridge); (c-d) skin membranes placed with dermis side down on a filter paper moistened with two-week skin culture medium and kept in freezer (-20 °C) for 6 weeks or 1 year (referred to as −20 °C or −20 °C 1y, respectively); and (e) skin membranes placed with dermis side down on a filter paper moistened with two-week skin culture medium and kept in freezer (-80 °C) for 6 weeks (referred to as −80 °C).

2.3. Solubility of methyl salicylate (MS) and acyclovir (AC)

To investigate how the solubility of each model drug was influenced by the presence of the other model drug, a combination of solubility experiments was performed. First, the solubility of MS and AC was investigated separately in both citrate buffer (CB, 130.9 mM NaCl, 2.5 mM $C_6H_8O_7$ and 7.5 mM $C_6H_5O_7Na_3$, pH 5.5) and phosphate buffered saline solution (PBS, 130.9 mM NaCl, 5.1 mM $Na_2HPO_4{\cdot}2H_2O$ and 1.5 mM KH_2PO_4, pH 7.4). Next, the solubility was determined in mixtures containing both drugs, either in CB or PBS. All solubility experiments were performed by adding the drug(s) in excess and stirred using a magnetic stirrer for 30 min at room temperature. Next, the saturated solutions were kept at 32 °C under 1 h. Finally, the solutions were centrifuged at 14,300 relative centrifugal force for 2x3 min before dilution and analysis.

2.4. Permeation of methyl salicylate (MS) and acyclovir (AC)

The skin membranes were taken out from each storage condition and allowed to thaw/equilibrate at room temperature. Thereafter, they were mounted in a Franz diffusion cell setup (PermeGear Inc., Hellertown, PA 18,055 USA) with an orifice area of 1.97 cm^2 and a receptor volume of 7 ml. The Franz cells were heated with a circulating water bath keeping the membrane temperature constant at 32 °C. The membranes were allowed to hydrate for 1 h with dermis side in contact with the receptor solution (i.e., PBS, pH 7.4), while epidermis was exposed to ambient air. Next, 2.5 ml of donor formulation was added, containing 1.5 mg/ml AC and 0.7 mg/ml MS mixed in CB (pH 5.5). In average, the decrease of the AC concentration in the donor phase after 24 h was <1 %, taking all experiments into account, which adequately satisfies infinite dose regime for this substance. However, the decrease of MS concentrations in the donor phase was significantly higher, corresponding to around 30 ± 5% after 24 h. For this reason, it was decided to compare the cumulative amount permeated after 8 h, at which the permeation was more or less linear (see Fig. S2J). Samples were taken from the receptor compartment in 1 h intervals between 1 and 8 h and after 24 h. After 24 h the skin was washed five times with citrate buffer, the cell flange was removed, and epidermis was gently separated from dermis using a scalpel. AC, MS, and SA (salicylic acid) were extracted from the skin tissue by sonication for 30 min in an extraction fluid consisting of 10% (v/v) acetic acid, 10% (v/v) acetonitrile and 80% (v/v) phosphate buffer (10 mM KH_2PO_4, pH 7.5).

2.5. Electrical impedance spectroscopy measurements

Electrical impedance spectroscopy (EIS) measurements were performed on the skin membranes using a four-electrode setup mounted in the same Franz diffusion cells as described in the previous section. A detailed description of the system can be found in our previous work (Björklund et al., 2013b). The electrodes were connected to a potentiostat from Ivium Technologies (Eindhoven, The Netherlands). EIS measurements were performed directly after addition of donor solution (t = 0 h) and after 24 h. Impedance data were collected in the frequency range from 1 Hz to 1 MHz with 5 frequencies per decade. The amplitude of the applied voltage was kept 10 mV to ensure low current densities in the lower frequency range. The reference and sensing electrodes were removed from the Franz diffusion cells between measurements to prevent KCl leakage.

2.6. Evaluation of electrical impedance data

The effect of the storage conditions on the electrical properties of the skin barrier was evaluated by means of skin membrane resistance, conductance and effective capacitance, which were derived from the data obtained from the EIS measurements according to previously described methods (Björklund et al., 2013b; Morin et al., 2020). In brief, the EIS measurements provide information about the electrical properties of the SC based on the relationship between the alternating voltage and the alternating current (Rosendal, 1945). The total impedance of skin ($|Z|_{skin}$) is dominated by the impedance of the *stratum corneum* (SC), especially in the lower frequency region (<1 kHz), and is directly correlated to the integrity of the skin barrier (Björklund et al., 2013b; Morin et al., 2020; Sekkat et al., 2002). Several equivalent circuits of varying complexities have been developed previously in an attempt to model the complex impedance of SC originated from its nonlinear (nonideal) properties (Kontturi and Murtomaki, 1994; Nolan et al., 1993; Pliquett and Pliquett, 1996; Yamamoto and Yamamoto, 1976). In this work, the electrical impedance was modelled using an electrical circuit consisting of a leading resistor for the electrolyte solution surrounding the skin membrane (R_{sol}) in series with a parallel combination of a resistor, representing the membrane resistance (R_{mem}), and a constant phase element (CPE) (Hirschorn et al., 2010; Kalia and Guy, 1995; Lackermeier et al., 1999). The CPE accounts for a nonlinear distribution of time constants caused by the heterogenous structure of SC and makes it possible to determine the effective capacitance (C_{eff}) of the skin membrane (Orazem et al., 2006). A detailed description of how to derive effective capacitance from the impedance data can be found elsewhere (Björklund et al., 2013b; Hirschorn et al., 2010). The R_{mem} was determined from the absolute impedance $|Z|_{skin}$ in the frequency range 1–10 Hz. The conductance of the skin was determined by taking the inverse of the real part of the impedance (Z_{Re}) at maximum phase shift, which for the majority of measurements occurred between 2.5 and 10 kHz. The reason for determining the conductance according to this protocol was to include a parameter that reflects high frequency conductance pathways of the skin barrier, as compared to the low frequency conductance given by the inverse of the low frequency parameter R_{mem}. All impedance data were normalized with the area of the skin membrane (1.97 cm^2) that is effectively probed by the EIS technique.

2.7. Analytical method

AC, MS, and SA were analyzed using a reverse phase high-pressure liquid chromatography (RP-HPLC) method with ultraviolet (UV) detection. A Dionex UltimMate 3000 (Thermo Fisher Scientific, Waltham, USA) instrument, equipped with a high-performance pump (LPG-3400S), diode array detector (DAD-3000), autosampler (ACC-3000), and column oven (TCC-3000SD), was used for the quantification. A Hypersil GOLD 150x4.6 mm 3 μm column (Thermo scientific, Göteborg Sweden) was used for separation, while a pre-column (SecurityGuard Ultra Cartridges UHPLC C18) was used to remove impurities from the skin membranes. The column temperature was kept constant at 23 °C and 50 μL was used as injection volume. Elution was carried out using a

gradient with phosphate buffer (10 mM KH_2PO_4, pH 7.5) and acetonitrile. The linear gradient started after 1 min and went from 5% (v/v) acetonitrile to 80% (v/v) acetonitrile in 7 min, whereafter isocratic elution was kept for 2 min. A flow rate of 1 ml/min was used. AC was monitored at 254 nm, while MS and SA were monitored 302 nm. Data evaluation was performed with Chromeleon 7.2 (Thermo Fisher Scientific, Waltham, USA).

2.8. Data analysis and statistics

Cumulative amounts of AC, MS and SA were calculated from the amounts (μg) permeated at each sampling interval and normalized by the surface area of the skin membrane (cm^2) available for permeation. The cumulative amounts of AC obtained after 24 h, MS and SA obtained after 8 h, as well as the amounts (μg/cm^2) of these compounds accumulated in epidermis or dermis over 24 h were considered for statistical analysis. For clarity, all data are compiled in the supportive information (see Fig. S1 for AC data, Fig. S2 and S3 for MS data, and Fig. S4 and S5 for SA data). The statistical analysis of all data was performed using R (R Core Team, 2020). Data are presented as bar plots showing mean values ± standard deviation (SD), determined from the number of observation (n) after removal of the extreme outliers using interquartile range (IQR) method. Extreme outliers are defined as values appearing above Q3 + 3 × IQR or below Q1 − 3 × IQR, where IQR is defined as IQE = Q3 - Q1, Q1 and Q3 are first and third quartiles respectively. In all cases, $n = 5$–8 after removal of extreme outliers. The normality of the data distribution was tested using Shapiro-Wilk and Kolmogorov-Smirnov tests as well as by examining quantile–quantile (QQ) plots. Equality of the variances within groups was assessed by performing Levene's test. The effect of storage conditions on the biophysical properties of the skin barrier was assessed by performing one-way ANOVA with planned contrast where fresh skin was used as a control and was compared to skin membranes stored at other conditions. Two-sample t-test was carried out to investigate the effect of storage time at −20 °C on the properties of the skin barrier (i.e., impedance and permeability) by comparing the results obtained from skin membranes used after 1 month of storage to the results obtained for skin membranes stored for 1 year. The relationship between skin permeability (cumulative amount) and skin impedance (R_{mem} and C_{eff}) was assessed by Spearman's rank correlation on the total dataset including all storage conditions. The significance levels used were: n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results and discussion

The aim of this work was to explore the effect of different storage conditions on skin permeability, biodistribution and skin metabolism, and the electrical properties of the skin barrier. Five different storage conditions were considered: a) 4 °C overnight (used as control and referred to as Fresh), (b) Fridge, (c) −20 °C for 6 weeks, (d) −20 °C for 1 year, (e) −80 °C for 6 weeks. The permeability of acyclovir (AC) and methyl salicylate (MS) across skin membrane was investigated in parallel with skin electrical impedance measurements over 24 h. Considering that MS is subject to ester hydrolysis into salicylic acid (SA), the concentration of SA was also determined. Sampling of receptor solution was performed once per hour during first 8 h, while a final sampling was performed after 24 h. EIS measurements were performed before (0 h) and after (24 h) the permeability experiments. After termination of the experiment, the skin membranes were washed with a buffer solution followed by separation of epidermis from dermis in order to determine the amount of each permeant accumulated in skin. Prior to performing these studies, the solubility of AC and MS in CB and PBS was determined to ensure that sink conditions prevailed during all experimental conditions, see Table 1.

Table 1
Solubility of acyclovir (AC) and methyl salicylate (MS) in citrate buffer (CB and phosphate buffered saline (PBS). Data are reported as mean ± SD ($n = 3$).

Sample	AC solubility (mg/ml)	MS solubility (mg/ml)
AC in CB	2.04 ± 0.05	–
AC in PBS	2.19 ± 0.03	–
MS in CB	–	0.77 ± 0.01
MS in PBS	–	0.79 ± 0.01
AC and MS in CB	2.10 ± 0.07	0.88 ± 0.00
AC and MS in PBS	2.22 ± 0.13	0.81 ± 0.01

3.1. The effect of different storage conditions on skin permeability and electrical impedance properties of the skin barrier

The impact of the different storage conditions on the investigated parameters is summarized in Fig. 1, where the results obtained with skin membranes stored overnight at 4 °C are referred to as Fresh and used as reference in comparison to the results obtained with the different storage conditions.

Starting with the relatively hydrophilic AC molecule, the cumulative amount permeated over 24 h is shown in Fig. 1A. The lowest permeation is observed in case of fresh skin (0.23 ± 0.30 μg/cm^2), which is significantly lower compared to the highest permeation obtained for skin membranes stored at −20 °C (1.95 ± 1.76 μg/cm^2). Storage of membranes in the fridge or at −80 °C results in similar values of the AC permeability and fall somewhere between the cases of fresh and −20 °C (Fig. 1 A). These results can be compared to similar studies using excised pig skin and relatively hydrophilic compounds (Meira et al., 2020; Sintov and Greenberg, 2014). For example, it has been shown that the permeability of caffeine (MW 194 g/mol, logP −0.63) across freeze stored (-20 °C for 1 week) pig skin was significantly increased as compared to fresh skin (Sintov and Greenberg, 2014). Further, the permeability of penciclovir (MW 523 g/mol, logP −1.4) was observed to be slightly higher (although not significantly) in the case of membranes stored for 30 days at −20 °C, as compared to fresh membranes (Meira et al., 2020). Taken together, even though the permeability of AC was only statistically lower across fresh skin, as compared to the permeability across skin membranes stored at −20 °C (Fig. 1A), a trend is discernable showing somewhat elevated fluxes also after fridge or freeze storage.

The partitioning of AC in the epidermis (Fig. 1B) is observed to be significantly elevated after freeze storage (both at −20 °C and −80 °C), while fridge-stored membranes show similar concentration, as compared to fresh membranes. Meira et al. employed pig skin and reported that the epidermal absorption of penciclovir (MW 523 g/mol, logP −1.4) was only slightly elevated after freeze storage at −20 °C for 30 days compared to fresh skin, while prolonged freeze storage for 60 and 90 days resulted in significantly elevated epidermal concentrations (Meira et al., 2020). Thus, the present results, in combination with previous studies, implies that freeze storage results in increased epidermal absorption of hydrophilic drugs, which may be a consequence of the enhanced skin permeability of hydrophilic drugs after freeze storage.

The dermal concentration of AC after 24 h exposure of the drug solution is observed to be unaffected by the different storage conditions (Fig. 1C). Further, when compared with the amounts absorbed into epidermis (Fig. 1B), the results show that the dermal concentrations are generally lower (Fig. 1C). This is in line with previous findings showing consistently lower dermal concentrations of penciclovir (MW 523 g/mol, logP −1.4), as compared to the epidermal absorption (Meira et al., 2020). However, these results are contrasting previous findings of caffeine partitioning, showing consistently higher amounts of caffeine recovered from the dermal compartment as compared to epidermis, irrespective of storage condition (Nielsen et al., 2011). This highlights the fact that it is difficult to find general partitioning profiles in different skin compartments even for drugs with similar physicochemical

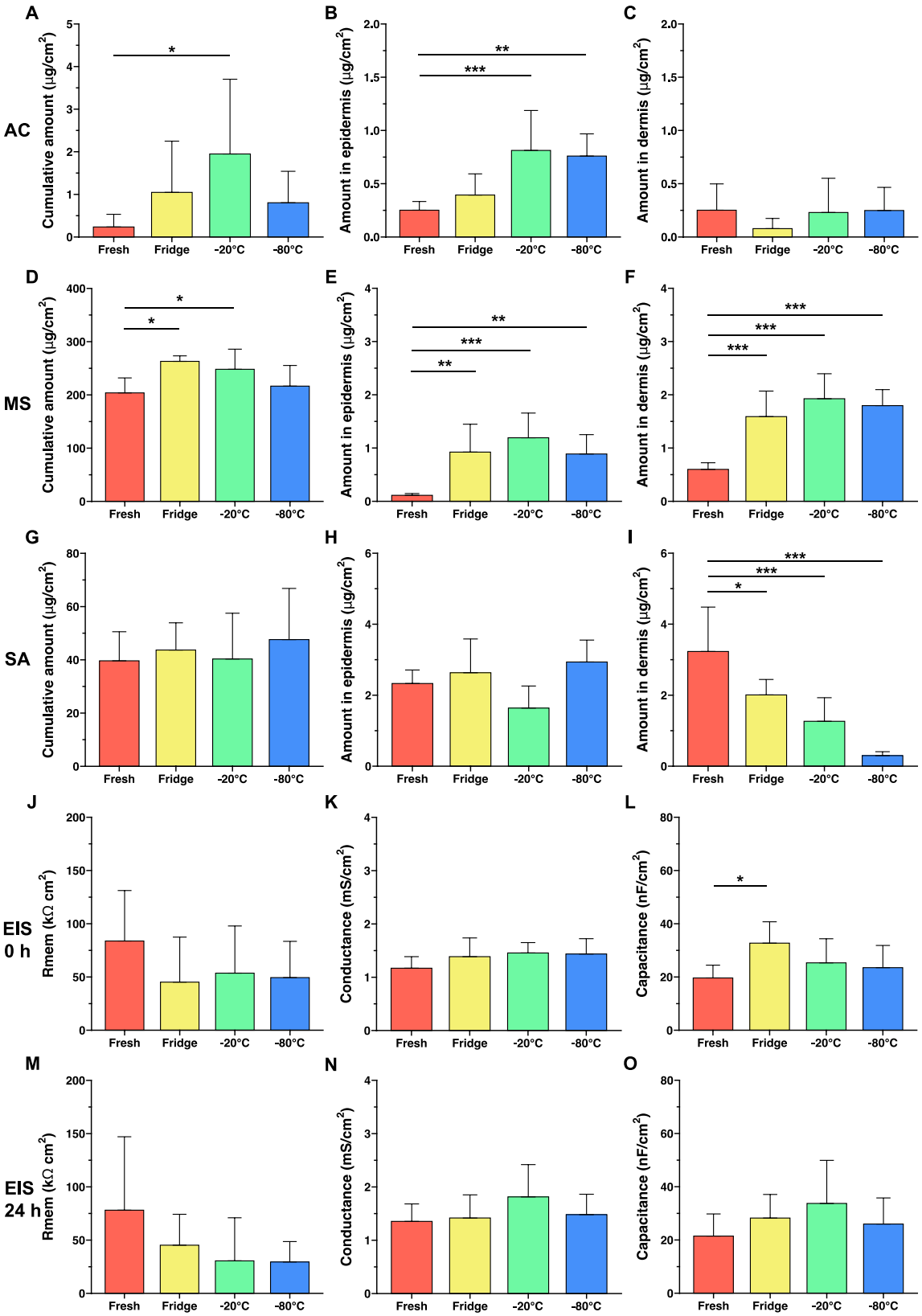

Fig. 1. The effect of the skin storage conditions on skin permeability, drug distribution in epidermis and dermis, and on the electrical impedance properties. (A, B, C) show results for acyclovir (AC), (D, E, F) show results for methyl salicylate (MS), (G, H, I) show results for salicylic acid (SA) after ester hydrolysis of methyl salicylate, (J, K, L) show initial values of skin membrane resistance, conductance, and effective capacitance, respectively, while (M, N, O) show the corresponding values obtained after 24 h. The significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

properties.

For the more lipophilic substance (i.e., MS), the permeability is at least two orders of magnitude greater as compared to AC under the current experimental conditions. The lowest transdermal delivery of MS is observed in case of fresh skin, which is significantly lower compared to permeation across membranes stored in fridge and at −20 °C (Fig. 1D). These results are in line with the observed lower permeability of AC in the case of fresh skin (Fig. 1A), implying that the general integrity of the skin barrier is superior in the case of fresh skin membranes. Further, both the epidermal and dermal partitioning of MS is significantly lower for the fresh membranes, as compared to the other storage conditions (Fig. 1E and F, respectively). Interestingly, the results in Fig. 1I clearly show that the dermal concentration of SA is significantly higher in the case of fresh skin, as compared to the other skin storage protocols. A reasonable explanation for these findings is that the MS ester is proficiently hydrolyzed into SA in the case of fresh skin. Effectively, this leads to reduced amounts of MS and increased amounts of SA inside fresh skin, which assumingly is associated with more preserved esterase activity. In support for this, the skin metabolism of MS into SA has previously been shown to occur both *in vitro* and *in vivo* by native esterase residing in the viable skin (Guzek et al., 1989; Potts et al., 1989). However, the effect of skin storage of MS skin metabolism is less studied, and the present findings clearly show that skin kept according to the various storage conditions results in significantly higher amounts of intact MS in the epidermis and dermis (Fig. 1E and F). This implies attenuated esterase activity due to skin storage and that fresh skin is associated with a higher level of esterase activity, particularly in the dermis.

Nevertheless, the cumulative amounts of SA ending up in the receptor medium (Fig. 1G), as well as the amounts of SA extracted from the epidermis (Fig. 1H), are similar for all conditions (i.e., no statistical difference between the storage conditions). These observations imply that the activity of esterase inside the skin tissue is maintained to some extent regardless of fridge or freeze storage. Speculatively, the fact that the SA concentration collected from the epidermis is more or less similar, irrespective of storage protocol (Fig. 1G), suggests that the activity of epidermal esterase is relatively unaffected by, e.g., freezing, which otherwise may be expected to result in enzyme deactivation. On the other hand, esterase located in the softer and more aqueous dermal compartment is seemingly severely affected by skin storage, in particular by freeze storage, effectively resulting in lower amounts of SA (Fig. 1I). In summary, these results illustrate that skin storage has a significant effect on the skin permeability of MS and also alters the local drug concentration in the epidermal or dermal compartments. This is an important finding that should be considered in relation to the purpose of the topical or transdermal drug formulation under investigation in similar *in vitro* studies.

The permeability data for AC (Fig. 1A) and MS (Fig. 1D) can be related to the electrical resistance of the skin membranes, which was measured initially (i.e., 0 h, Fig. 1J) and after 24 h (Fig. 1M). Notably, both for AC and MS, an opposite relationship is observed for these parameters. In other words, fresh membranes have the highest resistance towards transport of electrical charge carriers and the lowest AC and MS permeabilities, while skin storage results in lower electrical resistance values and higher permeability values in general.

The skin conductance is more or less similar for all storage conditions (Fig. 1K and 1 N). Considering that the conductance was determined from the real part of impedance in the high frequency region (i.e., between 2.5 and 10 kHz), it is likely that this parameter mainly reflects the hydration degree of the skin tissue, including pore filling by aqueous media of skin furrows and appendages (Morin et al., 2020). Thus, the skin conductance is expected to be similar in all cases considering that the skin membranes are immersed in aqueous solution during the measurements.

The skin capacitance (Fig. 1L and 1O) is related to the dielectric properties of the SC, such as the low conductive lipid matrix and lipid-protein domains, as well as charged lipid and protein species available for double-layer capacitance (Björklund et al., 2013b; Craane-van Hinsberg et al., 1995; Kalia and Guy, 1995; Kontturi and Murtomaki, 1994; Oh et al., 1993). The initial skin capacitance is observed to be significantly higher for the membranes stored in the fridge (i.e., storage for 4 days on wetted filter paper), as compared to the fresh membranes (Fig. 1L). This difference vanishes, however, after 24 h (Fig. 1O). This implies that the effective dielectric constant of the capacitive domains in SC is initially elevated after fridge storage, e.g., by an elevated degree of hydration. It has previously been shown that the effective capacitance of the SC membrane is higher in the case of a more hydrated SC tissue, which is also associated with more mobile lipid and keratin molecular segments, as well as higher permeability of MS (Björklund et al., 2010; Björklund et al., 2013a; Björklund et al., 2013b). In line with this, a closer analysis of the permeability data of MS show that the cumulative amount of MS permeated across membranes stored in the fridge is significantly higher as compared to the permeation across fresh membranes, in particular for the initial time points (see Fig. S2). Based on these observations, it is possible that the initial hydration state of the SC is elevated after fridge storage, leading to relatively higher skin permeability of MS. After prolonged immersion in the donor solution, however, it is reasonable to assume that any initial variations of the hydration degree disappear, which could explain the successively more similar amounts of MS permeation across fresh and fridge stored skin (Fig. S2). Freeze storage seems to result in a more pronounced increase of the skin membrane capacitance when comparing the initial capacitance (Fig. 1L) with the final values (Fig. 1O), while the capacitance for fresh skin membranes remained more or less unaltered and the capacitance for skin stored at 4 °C decreased. Nielsen et al. also observed a more pronounced increase in skin capacitance when comparing the initial and final values (48 h) for membranes stored for 3 weeks at −20 °C (from 33 nF/cm^2 to 101 nF/cm^2) and at −80 °C (from 41 nF/cm^2 to 75 nF/cm^2), as compared to fresh skin membranes (from 24 nF/cm^2 to 56 nF/cm^2) (Nielsen et al., 2011).

3.2. The influence of the storage time on the skin barrier

To investigate the effect of prolonged freeze storage, experiments were performed on skin membranes stored for one year at −20 °C and compared with the results obtained with skin membranes stored for 6 weeks at −20 °C. The results are presented in Fig. 2.

Starting with the results of AC, the data in Fig. 2A show that the skin permeability is increased after prolonged storage time, which is in agreement with previous findings of penciclovir (MW 523 g/mol, logP −1.4) permeation, showing that prolonged freeze storage (60–90 days at − 20 °C) resulted in significantly elevated fluxes (Meira et al., 2020). Fig. 2B shows that the AC concentration in epidermis is significantly reduced after prolonged storage (to levels similar to fresh skin, see Fig. 1B), while the dermal concentration is unaffected as shown in Fig. 2C (similar values as compared to all other storage conditions, see Fig. 1C). These results are in contrast to the findings of Meira et al., showing a consistent increase of epidermal and dermal accumulation of penciclovir as a function of freeze storage time (Meira et al., 2020). The reason for this discrepancy remains unclear.

The skin permeability of MS, as well as the amount of SA ending up in the receptor solution following ester hydrolysis, is more or less unaffected by prolonged storage time at −20 °C as shown in Fig. 2D and 2G, respectively. On the other hand, after prolonged freezing, the epidermal and dermal concentration of MS is significantly different (i.e., lower and higher, respectively) as compared to skin membranes stored for 6 weeks (Fig. 2E and 2F). At the same time, the amounts of SA collected from epidermis and dermis are significantly higher for the case of prolonged freeze storage (Fig. 2H and 2I). It is difficult to rationalize these observations in terms of altered skin metabolism of MS by esterase residing in these skin compartments. However, it is safe to conclude that esterase activity is preserved to some extent even after one year freeze storage at

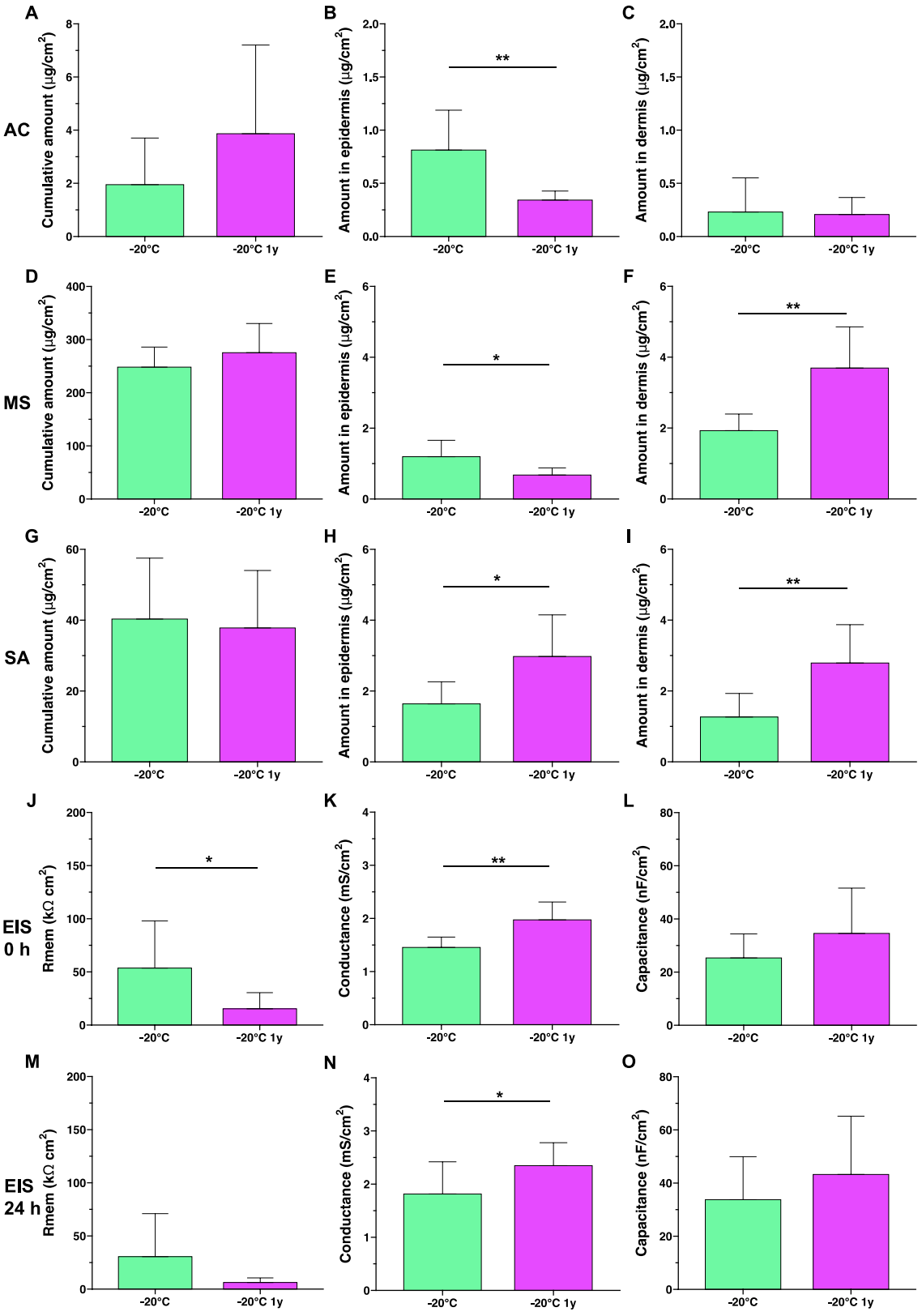

Fig. 2. The effect of the storage time at −20 °C on skin permeability, drug distribution in epidermis and dermis, and on the electrical impedance properties. (A, B, C) show results for acyclovir, (D, E, F) show results for methyl salicylate, (G, H, I) show results for salicylic acid (after ester hydrolysis of methyl salicylate), (J, K, L) show initial values of skin membrane resistance, conductance, and effective capacitance, respectively, while (M, N, O) show the corresponding values obtained after 24 h. The significance levels: * $p < 0.05$, ** $p < 0.01$.

−20 °C, which is an important finding.

Similar to the results presented in Fig. 1, the electrical resistance values presented in Fig. 2J (initial) and in Fig. 2M (final), are inversely related to the permeability of AC (Fig. 2A), showing that prolonged freeze storage results in reduced resistance towards electrical charge transport and increased AC permeability. Even though these are not statistically significant due to the large variability in the data (except for the initial resistance values, see Fig. 2J), the combination of data implies a general trend of successively debilitated skin barrier properties towards hydrophilic permeants. However, this effect is not observed in the permeation data of the more lipophilic MS, which remains unaffected after freeze storage at −20 °C for one year (Fig. 2D).

The skin conductance is observed to increase after prolonged freeze storage (Fig. 2K and 2 N), as well as the skin capacitance (Fig. 2L and 2O). These observations may imply that the overall tissue integrity is continuously being destabilized as a function of freeze storage time, giving rise to a weakened tissue that is more prone to swell, e.g., by inclusion of aqueous pools (Albér et al., 2013; Warner et al., 2003).

3.3. Correlation between permeability and electrical impedance properties of the skin

Great biological variation is associated with *in vitro* experiments on skin tissue, which should be considered in combination with the multitude of parameters that can be subject to variation during preparation and storage of skin tissue. Therefore, it seems reasonable to explore the possibility of finding correlations between *in vitro* permeability data (including epidermal and dermal drug accumulation data) and the parameters obtained by electrical impedance spectroscopy (EIS). For example, *in vitro* screening of skin permeability of various drugs, toxins, or other compounds of relevance will most likely continue to be performed with skin from various animal sources and prepared and stored by different protocols. In these cases, it would be helpful to have complementary data from individual skin membranes obtained with an alternative, but compatible, technique. Indeed, EIS represents a valid technique for this. To explore this topic, we screened all data obtained from the AC and MS permeability experiments, including the epidermis and dermis drug accumulation determinations, with the collected EIS data (i.e., the parameters presented in Figs. 1 and 2 for all collected data). The most promising results from this screening are presented in Fig. 3 (AC and MS permeability data versus skin membrane resistance) and Fig. 4 (AC and MS permeability data versus skin membrane effective capacitance). Notably, the correlations are performed on the complete collection of data from individual skin membranes, irrespective of storage condition.

As shown in Fig. 3, strong correlations are observed between the AC permeability and the electrical skin resistance data, both for the initial (Fig. 3A) and final (Fig. 3B) impedance data, with ρ of 0.84 and 0.82, respectively. Thus, the results imply that the skin electrical resistance can be used as a tool for normalizing permeability data of hydrophilic compounds, in accordance with previous findings (Tang et al., 2001), independently of how the skin membranes were stored. However, for the more lipophilic MS molecule, the skin resistance data is seemingly less correlated with the permeability values (Fig. 3C and 3D). Particularly for the membranes stored for one year in −20 °C, which have relatively low resistance values and large variations (Fig. 2 J and M), but still show very similar MS permeability values, compared to storage at −20 °C for 6 weeks (Fig. 2D). Further, only a moderate positive correlation is observed between the skin electrical resistance and the amount of SA collected after 8 h (Fig. 3E and 3F). This indicates that the permeation of MS is less sensitive for alterations of the skin tissue that influence the hydrophilic diffusion pathways of SC (i.e., diffusion pathways for AC and electrolytes). Instead, the permeation of MS is likely more dependent on the status of the continuous lipid matrix. The results in Fig. 4C and D support this suggestion, showing strong correlations between the MS permeation values and the initial (ρ of 0.79) and final (ρ of 0.83)

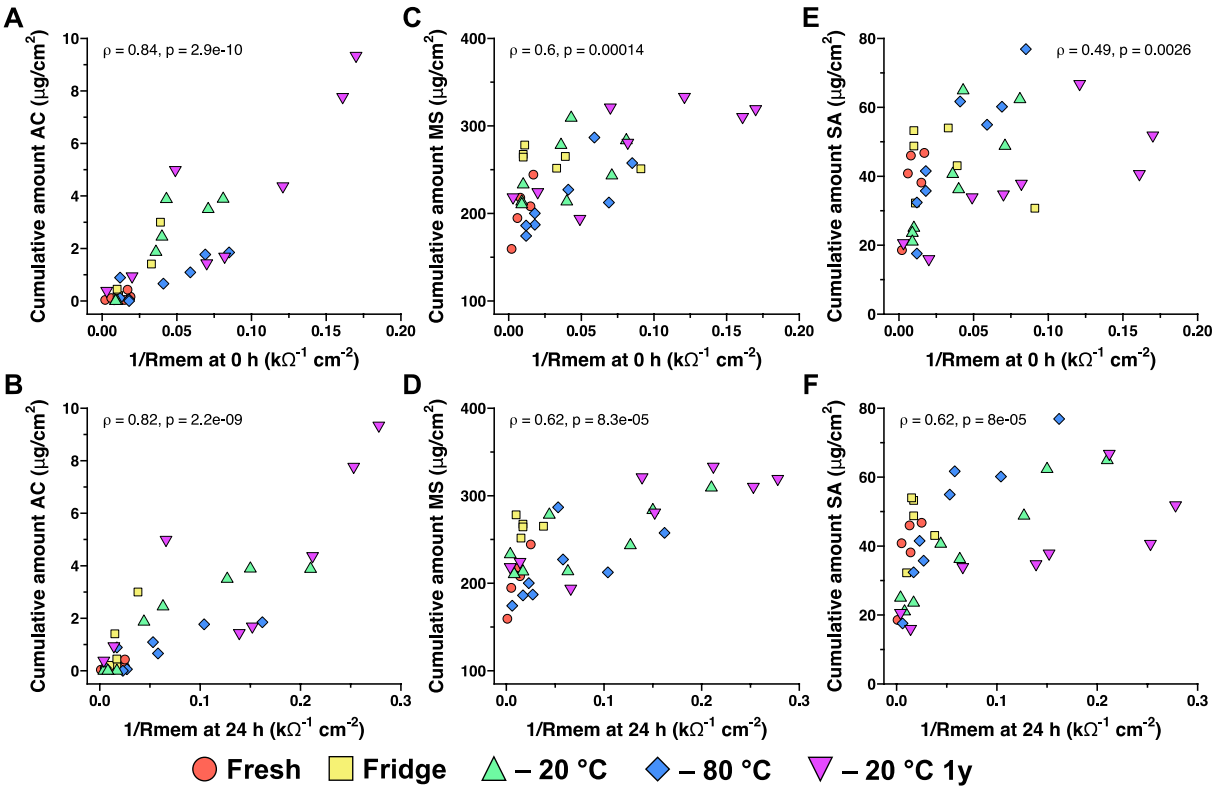

Fig. 3. Correlation between skin permeability and electrical skin resistance at time 0 h (upper panel) and after 24 h (lower panel). (A and B) show the cumulative amount of permeated acyclovir (AC), (C and D) show the cumulative amount of permeated methyl salicylate (MS), while (E and F) show cumulative amount of salicylic acid (SA) ending up in the receptor medium; all plotted as a function of $1/R_{mem}$.

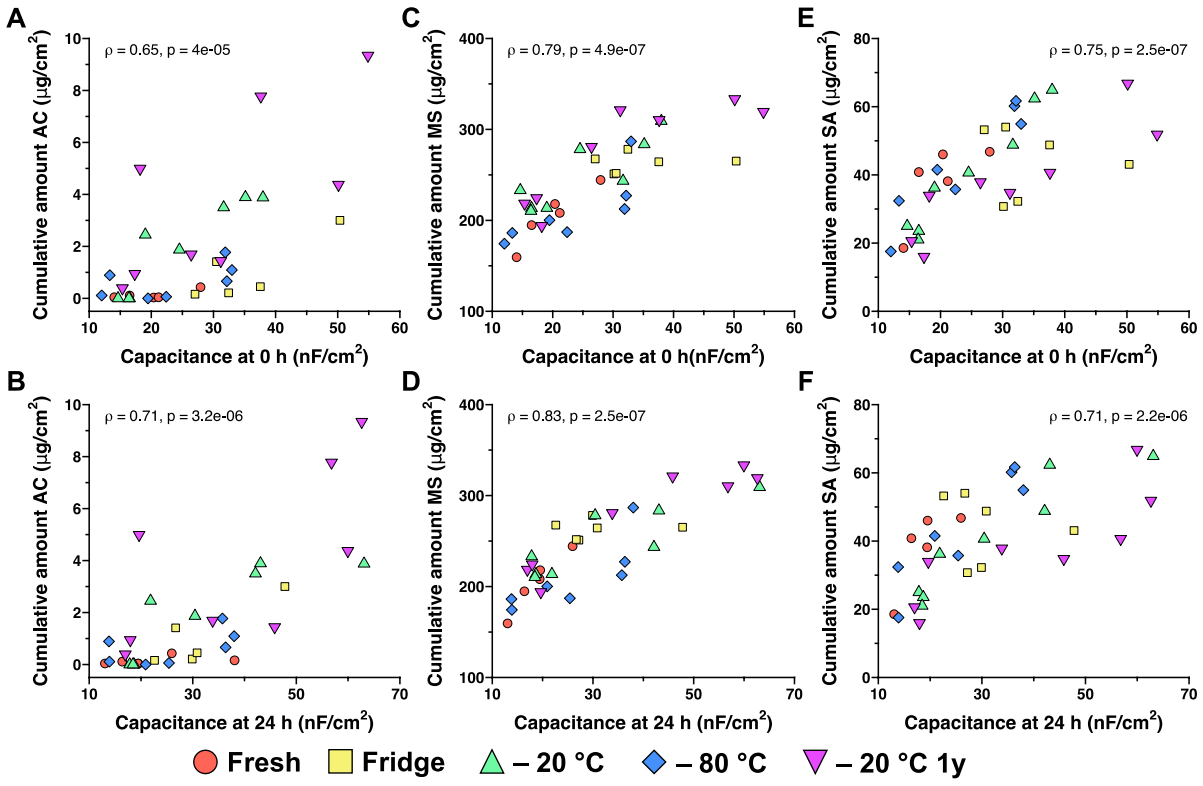

Fig. 4. Correlation between skin permeability and electrical skin capacitance at time 0 h (upper panel) and after 24 h (lower panel). (A and B) show the cumulative amount of permeated acyclovir (AC), (C and D) show the cumulative amount of permeated methyl salicylate (MS), while (E and F) show cumulative amount of salicylic acid (SA) ending up in the receptor medium; all plotted as a function of the electrical skin capacitance.

electrical skin capacitances. The skin capacitance is mainly attributed to the dielectric properties of the lipid matrix and lipid-protein domains of SC leading to restricted ion transfer, as well as limited ion transfer due to double layer charging of, for example, freely accessible charged amino acid residues of the keratin filaments (Björklund et al., 2013b; Craane-van Hinsberg et al., 1995; Kalia and Guy, 1995; Kalia et al., 1996; Kontturi and Murtomaki, 1994; Oh et al., 1993). As shown in Fig. 4E and F, the correlations between the cumulative amounts of SA and the skin capacitance values are also strong ($\rho > 0.7$). This can be rationalized by the observed increased MS permeation, leading to higher abundance of MS molecules available for ester hydrolysis into SA. For the more hydrophilic AC substance, on the other hand, the corresponding correlations are weaker (Fig. 4A and 4B), which is in line with the suggestion that this substance diffuses via an alternative pathway across SC. Taken together, these correlations can be used to improve and standardize interpretation of *in vitro* permeation data, where skin resistance is a helpful parameter for hydrophilic drugs, while the skin capacitance is useful for lipophilic substances. As such, EIS can be used to improve the analysis and enable comparisons between permeability results obtained in different laboratories and with skin stored at different conditions.

4. Conclusions

The influence of skin storage conditions on the *in vitro* skin permeability represents an issue with great complexity and, as previously suggested, it seems sensible for individual laboratories to test their own protocols of storage conditions (Barbero and Frasch, 2016). The present investigation of the effect of various skin storage protocols on the skin permeability of acyclovir and methyl salicylate, including the effect on the skin metabolism of the latter substance, contributes with new findings. In particular, by utilizing electrical impedance spectroscopy, complementary information on the skin membrane electrical properties is obtained. Based on the present results, we conclude following.

- Fresh skin shows lower permeability of the hydrophilic model drug acyclovir and higher electrical resistance, as compared to stored skin. This implies that the skin barrier towards hydrophilic substances and charge carriers is compromised to some extent during fridge and freeze storage. In particular, the skin permeability of acyclovir across fresh skin is significantly lower as compared to skin stored at −20 °C.
- The epidermal and dermal drug distribution for acyclovir is significantly affected by fridge and freeze storage, leading to higher acyclovir concentrations in these compartments as compared to the corresponding accumulation in fresh skin.
- The permeability of the lipophilic model drug methyl salicylate is concluded to be lowest for fresh skin, while fridge and freeze storage (-20 °C) leads to increased permeability.
- The significantly lower amounts of methyl salicylate detected in the epidermis and dermis of fresh skin, as well as the increased amounts of salicylic acid in the dermis, strongly suggest that higher metabolic esterase activity is maintained in fresh skin, effectively reducing the amounts of methyl salicylate via hydrolysis into salicylic acid.
- Although the hydrolysis of methyl salicylate is observed to be higher for fresh skin, the degradation of this ester is concluded to also occur in fridge and freeze stored skin, implying that esterase activity is maintained to some extent irrespective of storage condition (i.e., the metabolic activity is not zero).
- Strong correlations are observed between the acyclovir permeability and the electrical skin membrane resistance irrespective of storage condition.
- Strong correlations are shown between the methyl salicylate permeability and the skin membrane effective capacitance, which is related to the dielectric properties of the SC lipid and protein components.
- Electrical impedance spectroscopy represents a valuable tool for obtaining complementary information of skin membrane resistance and effective capacitance. In particular, the combination of EIS and

permeability data allow us to suggest that acyclovir (hydrophilic) and electrical charge carriers are transported via a hydrophilic pathway across the skin barrier and that their permeation routes are influenced by the skin storage protocol in a similar manner. On the other hand, the permeability of methyl salicylate is suggested to be more dependent on the status of the continuous lipid matrix, which is seemingly less affected by the various skin storage conditions.

Funding

This work was conducted within the research network Biobarriers - Health, Disorders and Healing funded by the Knowledge Foundation, Sweden (grant number 20190010).

CRediT authorship contribution statement

Maxim Morin: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. **Anna Runnsjö:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Tautgirdas Ruzgas:** Conceptualization, Funding acquisition, Methodology, Writing – review & editing. **Johan Engblom:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. **Sebastian Björklund:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2023.122891.

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