J. Pharm. Pharmacol. 1992, 44: 387–390
Received June 18, 1991

Effect of Freezing on Iontophoretic Transport Through Hairless Rat Skin

BOKA W. HADZIJA, STEPHEN B. RUDDY AND E. SCOTT BALLENGER

Division of Pharmaceutics, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA

Abstract—The influence of frozen storage of hairless rat skin on the constant-current iontophoretic transport of a model weak acid, salicylic acid, was examined. Nearly a threefold increase was observed in the steady-state flux of salicylic acid through previously frozen skin (217·1 nmol h^{-1} cm^{-2}) when compared with fresh, excised skin (72·3 nmol h^{-1} cm^{-2}). The iontophoretic permeability of skin to salicylic acid was also found to be dependent upon the length of time skin samples remained in the frozen state. Differential scanning calorimetry studies revealed distinct differences in the thermograms for fresh and frozen skin. These results suggest that storage of skin samples in the frozen state may contribute to physical or chemical changes in skin structure.

Transdermal iontophoresis may be described as a process in which drug molecules experience electrically assisted movement into and through the skin following application of an electrical potential difference. Present widespread interest in iontophoretic delivery is largely based upon the potential ability of this technique to overcome certain limitations routinely encountered during conventional transdermal drug delivery, enabling compounds of high molecular weight and considerable polar character to be administered via this route. Although the mechanisms responsible for iontophoretic transport are not fully understood, it is apparent that the physicochemical properties of both the skin and the permeant will likely influence the transport process (Bellantone et al 1986; Burnette & Ongpipattanakul 1987; Behl et al 1989; Lelawongs et al 1989). However, limited attention has been devoted to factors which may affect the inherent permeability of mammalian skin, particularly those related to the handling and storage of excised skin samples before use in in-vitro studies (Kasting & Bowman 1990a, b).

The skin may be envisaged as a complex, heterogeneous biomembrane consisting of 15–20% lipids, 40% proteins (primarily keratin) and 40% water (Tyle 1986; Chien et al 1989). Accordingly, skin specimens should be stored in such a way that the structure and integrity of the tissue remains unchanged. Although the effects of preserving the skin in the frozen state (generally at or below −20°C) are not immediately obvious, the possibility that the molecular arrangement of the skin's components may be permanently altered during the freezing process should not be ruled out.

While there is evidence to suggest that the freezing of excised human skin significantly alters the passive transport of chromone acids (Swarbrick et al 1982), other investigators have shown that no changes in the passive permeability of water through the skin occur following prolonged frozen storage (Astley & Levine 1976; Harrison et al 1984). Bronaugh et al (1986) found no significant difference in water permeability through fresh skin and skin frozen for a few days. However, apparent alteration of the barrier properties of the skin was observed for some specimens following frozen storage for longer periods of time.

Kasting & Bowman (1990a, b) recently compared the direct-current electrical properties of fresh and previously frozen human allograft skin. These investigators found that fresh skin is considerably less conductive than frozen skin at low current densities, and is more permselective to sodium ions than chloride ions during iontophoresis. In addition, dissimilarities were noted in the current-voltage properties of fresh and frozen tissue. However, it was not determined whether these observed differences result in enhanced permeability of drug molecules through frozen skin relative to fresh, excised tissue. Accordingly, the objective of this research was to examine the effects of frozen storage on the iontophoretic transport of a model compound, salicylic acid.

Correspondence: B. W. Hadzija, Division of Pharmaceutics, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA.

Materials and Methods

Materials

Salicylic acid was obtained from Sigma Chemical Company (St Louis, MO), and was used as received. All other chemicals were ACS reagent grade, while solvents were HPLC grade (Fisher Scientific, Fair Lawn, NJ). Aqueous solutions were prepared in deionized, purified water (Milli-Q Water Purification System, Millipore Corporation, Bedford, MA).

Preparation of skin specimens

Full-thickness skin was excised from hairless rats (male, retired breeders, 400–500 g, Charles River Laboratories, Raleigh, NC) following death by CO_2 asphyxiation. Upon harvest, the skin was removed of all subcutaneous fat and connective tissue. Skin slabs (2·5 × 2·5 cm) were cut from the dorsolateral region of three animals, providing 8 specimens from each animal. The 24 specimens were subsequently divided into 4 groups of 6 slabs (2 from each animal), after which each group was sealed in an inner cellophane layer and wrapped in two outer layers of aluminium foil. One group was temporarily refrigerated overnight (4°C), while each of the remaining groups was frozen (−20°C) for 3, 6 or 9 weeks.

Iontophoretic transdermal permeation system

The iontophoretic transdermal permeation system consisted of a stainless steel platform housing 18 magnetic stirring plates (600 rev min^{-1}) and 9 pairs of custom-made vertical diffusion cells. The donor and receptor chambers possessed volumes of 3·5 and 10 mL, respectively, while the area available for transport was 2·0 cm^2. The cells were maintained at 37°C by a circulating water bath (VWR Scientific, Philadelphia, PA). The power source employed in the study was custom-made (30 V, range 0·01 to 10·00 mA), and delivered constant current with an accuracy of 0·01 mA at 1·00 mA. Electrodes were constructed from platinum wire (99·9% pure, Fisher Scientific, Fair Lawn, NJ) and measured 0·25 × 24 mm.

Measurement of salicylic acid transport

At the designated time, each group of skin slabs was removed from the freezer and was permitted to thaw for a short time until the skin became pliable (1 h). After securing each specimen between the two halves of a diffusion cell, donor and receptor chambers were filled with isotonic Sørensen's buffer (pH = 7·4) to permit the skin to reach equilibrium hydration. At the end of 1 h, the contents of each chamber were discarded. Receptor chambers were immediately refilled with fresh Sørensen's buffer, while donor chambers were filled with a buffered solution of salicylic acid (15 mM, pH = 6·35). Electrodes were then immersed in the contents of each half cell, taking care not to make contact with the skin surface. The poles were oriented such that the cathode was contained within the donor chamber and the anode was contained within the receptor chamber. A constant current (0·16 mA cm^{-2}) was then initiated. At the conclusion of each 2 h interval, the entire volume of each receptor chamber was removed and replaced with fresh buffer. Samples were stored in polypropylene test tubes at 4°C for analysis by HPLC. In order to prevent significant fluctuations in donor pH, μL amounts of 5 M NaOH were added at 3 and 6 h. The transport studies were conducted over an eight hour period, after which the diffusion cells were disassembled and thoroughly cleaned.

Sample preparation and HPLC analysis

Before analysis, each aqueous sample was clarified with a 0·45 μm cellulose acetate syringe filter. A sample volume of 500 μL was then added to a clean polypropylene test tube, followed by addition of 100 μL of an internal standard solution (vanillin, 5 μg mL^{-1}). The contents of the tube were then vortexed for 30 s. Following complete mixing, a volume of 200 μL was removed for injection onto the column. Quantitative determination of salicylic acid was carried out by reversed phase HPLC. The basic system consisted of an M45 Solvent Delivery System (Waters Associates, Inc., Milford, MA), a Spectroflow 773 ultraviolet absorbance detector (ABI Analytical, Ramsey, NJ), a Chromatopac C-R6A (Shimadzu Corporation, Kyoto, Japan), and an Alltech Econosphere C18 (5 μm, 4 mm × 25 cm) column (Alltech Associates Inc., Deerfield, IL). Chromatographic conditions were as follows: solvent flow rate, 1·0 mL min^{-1}; analytical wavelength, 240 nm; operating pressure, 2500 psi; mobile phase, methanol:acetonitrile:water:85% phosphoric acid (360:91:48:1). Retention times of salicylic acid and vanillin were 6·0 and 3·3 min, respectively. Peaks of interest were well separated from those of skin contaminants.

Preparation of stratum corneum samples

Full-thickness specimens of hairless rat skin, fresh and previously frozen for 3 weeks, were submerged in purified water at 60°C for 2 min in order to isolate the epidermal layer of the skin. Following mechanical separation, the epidermis was soaked in a 2% w/v solution of Na-EDTA at 37°C for 1 h. Specimens were then placed on a smooth surface, stratum corneum side down, after which a sharp blade was used to remove the lower layers of epidermal tissue. Resulting stratum corneum sheets were repeatedly rinsed with deionized, purified water and spread on filter paper. Samples were subsequently dried over anhydrous $CaSO_4$ for 48 h in a desiccator before analysis by differential scanning calorimetry (DSC).

Differential scanning calorimetry

Samples of dried stratum corneum (13·25 mg) were thermally analysed using DSC (Perkin-Elmer DSC-4, Norwalk, CT). Scanning was performed at 20°C min^{-1} over a broad temperature range (0–190 °C) at a sensitivity of 10 mCal s^{-1}. Upon cooling, a second heating of each sample was performed.

Results

Iontophoretic transport of salicylic acid

The rates of iontophoretic transport of salicylic acid through

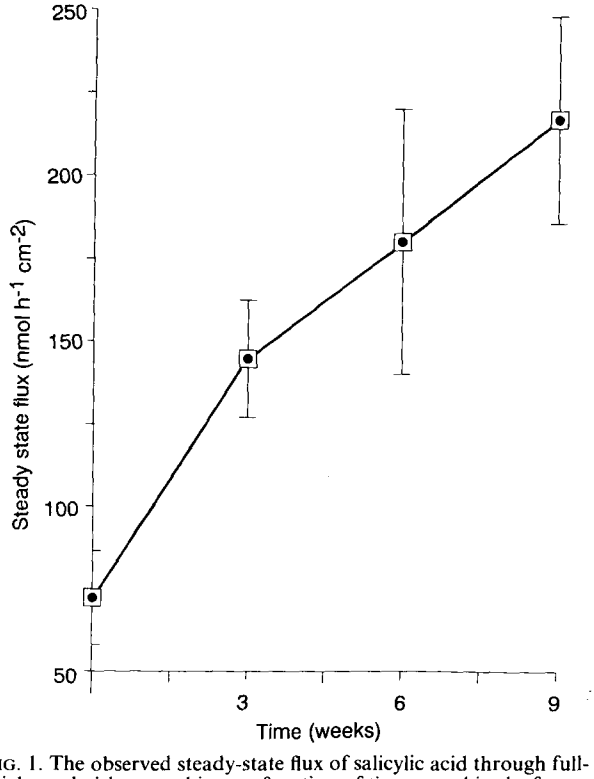

FIG. 1. The observed steady-state flux of salicylic acid through full-thickness hairless rat skin as a function of time stored in the frozen state. Error bars represent the standard error of the mean (n = 6). Current density = 0·16 mA cm^{-2}. Zero time corresponds to fresh, excised specimens.

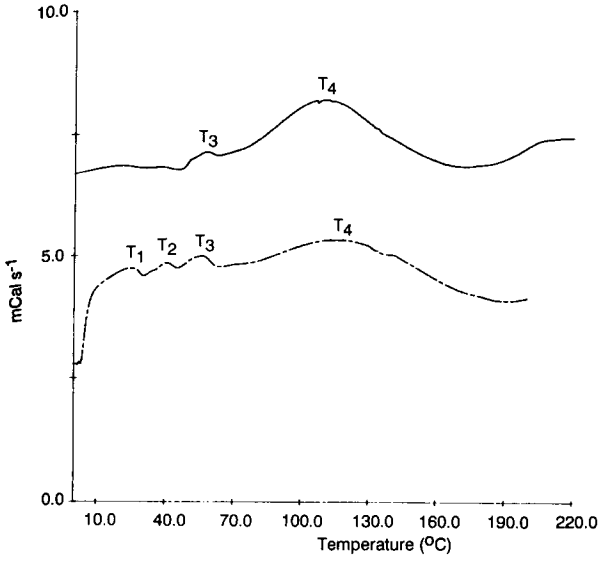

FIG. 2. DSC thermograms generated for fresh (lower profile) and previously frozen (upper profile) hairless rat stratum corneum. Thermal analysis was performed at 20°C min^{-1}.

skin were obtained from a plot of the cumulative amount of the drug transported per unit area as a function of time. Steady-state flux was determined by a least squares linear regression analysis of the last three data points (4, 6 and 8 h) for each group. The results clearly indicate that the rate of iontophoretic transport of salicylic acid through previously frozen skin was greater than through freshly excised skin from identical animals (Fig. 1). Iontophoretic permeability of salicylic acid through skin was smallest in fresh tissue (72·3 nmol h^{-1} cm^{-2}, $r^2 = 0{\cdot}999$), and increased sharply for skin frozen for 3 weeks (144·7 nmol h^{-1} cm^{-2}, $r^2 = 0{\cdot}998$), 6 weeks (180·1 nmol h^{-1} cm^{-2}, $r^2 = 0{\cdot}996$) and 9 weeks (217·1 nmol h^{-1} cm^{-2}, $r^2 = 0{\cdot}994$). A one-tailed Student's *t*-test confirmed that the transport rates were significantly different for all pairwise comparisons ($P < 0{\cdot}05$).

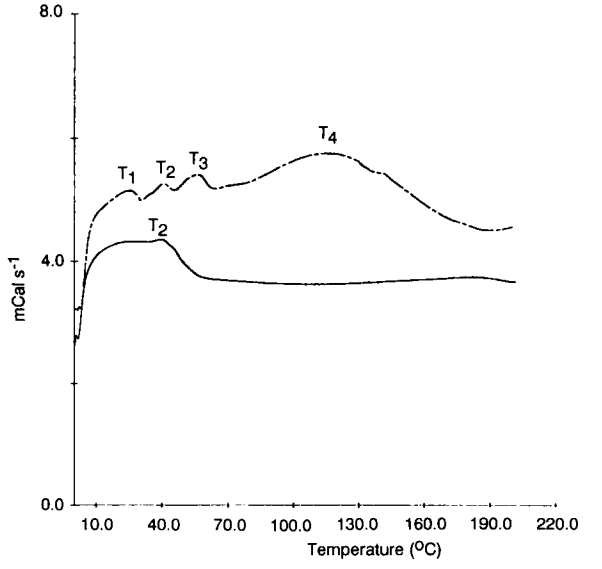

FIG. 3. DSC thermograms generated from the first heating (upper profile) and second heating (lower profile) for fresh hairless rat stratum corneum. Thermal analysis was performed at 20°C min^{-1}.

Thermal analysis of stratum corneum

Fig. 2 shows the DSC thermal transitions generated for fresh (lower profile) and previously frozen (upper profile) rat stratum corneum. A comparison of the two thermograms demonstrates similar behaviour at temperatures above 50°C, but marked differences at lower temperatures. A scan of fresh stratum corneum revealed 3 distinct peaks (T_1–T_3) between 25 and 60°C, while thermal analysis of previously frozen stratum corneum resulted in only one transition (T_3) in the same region. Both profiles were also characterized by a 4th broad transition (T_4) around 110°C. Upon cooling and reheating the fresh sample, only the peak at 40°C (T_2) was found to be reversible (Fig. 3).

Discussion

When fresh, excised, mammalian skin is rapidly frozen in an uncontrolled manner, the formation of ice crystals within the tissue is likely to result in permanent damage, thereby increasing the skin permeability to a variety of compounds. Accordingly, human skin banks generally take precautions to minimize such effects. Specimens are typically stored in a cryogenic medium (e.g. 10% glycerol) and are slowly frozen in a well-controlled environment (Kasting & Bowman 1990a,b). Since the procedure for freezing skin specimens in the present study did not include such measures, it was not surprising to find that the iontophoretic permeability of salicylic acid through frozen skin was significantly greater than through fresh tissue. However, the formation of crystalline water within the skin is not likely to account for the observed time-dependent increase in salicylic acid permeability over a nine-week period, indicating that an additional mechanism may be responsible for the subsequent changes in skin permeability.

Thermal analysis of mammalian stratum corneum by DSC has been widely used to detect structural anomalies associated with lipid and protein constituents (van Duzee 1975; Rehfeld & Elias 1982; Knutson et al 1985; Golden et al 1986; Goodman & Barry 1986; Khan & Kellaway 1989). Such alterations may be detected by examining thermal transitions representative of the "melting" of various structural domains, and may be associated with specific lipid or protein components based upon the temperature at which the transition occurs. Accordingly, results obtained from the present thermal analysis suggest that the time-dependent increase in the iontophoretic permeability of salicylic acid may be related to changes in stratum corneum lipid organization.

The primary differences noted in the thermograms of fresh and previously frozen stratum corneum correspond to low temperature peaks (T_1 at 25°C and T_2 at 40°C), which have been attributed to lipid transitions (van Duzee 1975; Knutson et al 1985; Golden et al 1986). In particular, Rehfeld et al (1981) have shown that low temperature transitions near 35°C are due to the presence of hydrophobic neutral lipids, and have additionally suggested the importance of these components in preserving the cohesive properties of the

stratum corneum. Accordingly, the observed differences in the low temperature thermal transitions for fresh and previously frozen skin indicate that the freezing process may adversely affect the structural organization of neutral lipids within the stratum corneum, thereby compromising its barrier properties. Francoeur et al (1990) recently suggested that solid-fluid phase separation within stratum corneum lipids may be responsible for enhancement of skin permeability following treatment with oleic acid. Although a plausible mechanism cannot be offered without conducting additional studies, one should not discount the possibility that the freezing process may exert a similar effect.

Alternatively, no observable differences were noted with respect to the transition at 60°C (T_3) or the high temperature transition at 110°C (T_4), which most likely correspond to other lipids and the denaturation of intracellular keratin, respectively. Consistent with the findings of Golden et al (1986), the high temperature transition was not found to be reversible upon reheating (Fig. 3). Based on these results, it is likely that stratum corneum proteins are not adversely affected during frozen storage.

The present results suggest that the storage of mammalian skin in the frozen state may result in physical and/or chemical changes in the tissue. However, it should be emphasized that such findings do not preclude the use of previously frozen specimens in iontophoretic transport studies, since the manner in which skin specimens are frozen may dictate the severity (or lack) of tissue damage. While we speculate that the extent of initial damage may be dependent upon pretreatment of skin specimens and the rate in which the freezing process proceeds, a definitive statement cannot be made based upon the present results. Furthermore, we surmise that putative time-dependent changes in stratum corneum lipids may be influenced by both the length of time in the frozen state and the temperature maintained throughout the storage process. Obviously, additional studies will be required to confirm these interpretations.

While the present findings demonstrate the effect of frozen storage on the iontophoretic permeability of a model weak acid through skin, similar studies employing different model permeants and/or animal models as well as alternative freezing procedures may yield different results. Nevertheless, the present results suggest that caution should be exercised when using skin specimens previously stored in the frozen state, and further advocate the use of screening procedures in order to properly validate the use of frozen skin specimens in iontophoretic transport studies.

Acknowledgements

This work was supported by Sterling Research Group, Great Valley, PA and Chugai-Upjohn Inc., Rosemont, IL. S. B. Ruddy is supported by a PMA Fellowship for Advanced Predoctoral Training in Pharmaceutics. The excellent technical assistance of Mrs Susan Gladfelter was greatly appreciated.

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